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(54) Title: COMPOSITIONS AND TREATMENTS FOR PNEUMONIA IN ANIMALS

(57) Abstract

New proteins and subunit antigens from *P. haemolytica* for use in stimulating immunity against respiratory diseases such as pneumonia, including shipping fever pneumonia, are disclosed. The subunit antigens include immunogenic amino acid sequences of *P. haemolytica* fimbrial protein, *P. haemolytica* plasmin receptor protein, and *P. haemolytica* 50 K outer membrane protein and *P. haemolytica* leukotoxin. The antigens can be used in a vaccine composition, either alone or in combination. Also disclosed are methods of vaccination as well as methods of making the subunit antigens employed in the vaccines.

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COMPOSITIONS AND TREATMENTS FOR PNEUMONIA IN ANIMALS

Description

Technical Field

The present invention relates generally to subunit antigens, vaccine compositions, and methods of administering the same. More particularly, the present invention relates to Pasteurella haemolytica proteins for use in stimulating immunity against pneumonia.

Background of the Invention

Respiratory disease affecting feedlot cattle causes tremendous losses yearly to the cattle industry. Calves are the most severely affected, and a large number of these calves die. This disease is associated with pathogenic microorganisms, particularly Pasteurallae species, and various stresses, such as transportation and overcrowding.

Shipping fever is the most economically important respiratory disease associated with Pasteurella species. The disease is characterized by sudden onset, usually within two weeks of stress. The symptoms include dyspnea, cough, ocular and nasal discharge, inappetance

and rapid weight loss, fever, increased lung sounds, immunosuppression, general depression, viral and/or bacterial infection of the lungs. Various bacteria and viruses have been isolated from affected animals including Pasteurella spp., bovine herpes virus 1, parainfluenza-3 virus, bovine respiratory syncytial virus and Mycoplasma species. The disease typically affects 15-30% of exposed animals and the resulting deaths are typically 2-5% of the exposed population.

Exposure of the animal to stress, plus infection with a variety of viruses, as described above, appears to make the animal susceptible to fibrinous pneumonia caused by P. haemolytica, and to a lesser extent, Pasteurella multocida. For a general background on shipping fever see Yates, W.D.G. (1982) Can. J. Comp. Med. 46:225-263.

P. haemolytica also causes enzootic pneumonia and can infect a wide range of animals, in addition to cattle, including economically important species such as sheep, swine, horses and fowl. P. haemolytica is also frequently found in the upper respiratory tract of healthy animals. Pneumonia develops when the bacteria infects the lungs of these animals. Protection against Pasteurella-associated diseases is therefore economically important to the agricultural industry.

There are two known biotypes of P. haemolytica designated A and T. There are also 12 recognized serotypes which have been isolated from ruminants. Biotype A, serotype 1 (referred to hereinafter as "A1") predominates in bovine pneumonia in North America. Shewen, P.E. and Wilkie, B.N. (1983) Am. J. Vet. Res. 44:715-719. However, antigens isolated from different serotypes appear to be somewhat cross-reactive. See, e.g., Donanchie et al. (1984) J. Gen. Micro. 130:1209-1216.

Previous Pasteurellosis vaccines have utilized whole cell preparations of either live or heat killed

bacteria of various serotypes as described in U.S. Patent Nos. 4,328,210, 4,171,354, 3,328,252, 4,167,560 and 4,346,074. Traditional vaccine preparations, however, have not been effective in protecting against Pasteurella infections. Indeed, vaccinated animals are frequently more susceptible to the disease than their non-vaccinated counterparts. Martin et al. (1980) Can. J. Comp. Med. 44:1-10. The lack of protection offered by traditional vaccines is probably due to the absence of important antigens, virulence determinants, or the presence of immunosuppressive components in the preparations.

Other vaccine preparations have included crude supernatant extracts from P. haemolytica. See, e.g., Shewen, P.E. and Wilkie, B.N. (1988) in Can. J. Vet. Res. 52:30-36. These culture supernatants, however, contain various soluble surface antigens of the bacterium and produce variable results when administered to animals. Other preparations include capsular extracts obtained via sodium salicylate extraction (See, e.g., Donanchie et al. (1984) 130:1209-1216; U.S. Patent No. 4,346,074), saline extracted antigens (See, e.g., Lessley et al. (1985) Veterinary Immunology and Immunopathology 10:279-296; Himmel et al. (1982) Am. J. Vet. Res. 43:764-767), and modified live Pasteurella mutants.

Still other attempts at immunization have included the use of a purified cytotoxin from P. haemolytica. See, e.g. Gentry et al. (1985) Vet. Immunology and Immunopathology 9:239-250. This cytotoxin, which is a leukotoxin, is secreted by actively growing bacteria. Shewen, P.E., and Wilkie, B.N. (1987) Infect. Immun. 55:3233-3236. The gene encoding this leukotoxin has been cloned and expressed in bacterial cells. Lo et al. (1985) Infect. Immun. 50:667-671. Calves which survive P. haemolytica infections possess toxin-neutralizing anti- body. Cho, H.J. and Jericho, K.W.F. (1986) Can. J. Vet.

Res. 50:27-31; Cho et al. (1984) Can. J. Comp. Med. 48:151-155.

Electron microscopy of intact P. haemolytica A-1 cells has demonstrated the presence of two types of fimbriae. Morck et al. (1987) Can. J. Vet. Res. 51:83-88. One type is rigid and easily sheared from the cell while the other is thin and flexible. The purpose of these fimbriae has not yet been determined. For some bacteria, however, fimbriae play a role in infection. See e.g. Normark et al. (1986) in Protein-carbohydrate Interactions in Biological Systems (D. Lark ed., 1986) pp. 3-12; Mooi, F. and deGraaf, F.K. (1985) Curr. Top. Microbiol. Immunol. 118:119-136.

Group A streptococci have recently been shown to possess a surface receptor that binds to host cell plasmin but not its precursor, plasminogen. Lottenberg et al. (1987) Infect. Immun. 55:1914-1928; Broeseker et al. (1988) Microbial Pathogenesis 5:19-27. Plasmin is a protease capable of hydrolyzing fibrin, extracellular matrix proteins and several plasma proteins. Therefore, it may be an important bacterial virulence mechanism and a potential immunogen.

Disclosure of the Invention

It has been discovered that subunit vaccines based on selected cell surface antigens isolated from P. haemolytica protect cattle from respiratory diseases such as pneumonia, including shipping fever pneumonia, caused by this bacterium. These subunit vaccines appear to be substantially more protective than prior art vaccines. Furthermore, these subunit vaccines can be produced using recombinant DNA technology or by chemical extraction. Based on these discoveries, the present invention can take several embodiments.

In one embodiment, the present invention is directed to a vaccine composition including a pharmaceuti-

cally acceptable vehicle and a subunit antigen composition comprising at least one immunogenic polypeptide comprising an immunogenic amino acid sequence of a P. haemolytica protein, or an amino acid sequence substantially homologous and functionally equivalent thereto, selected from the group consisting of P. haemolytica fimbrial protein, P. haemolytica plasmin receptor protein, P. haemolytica 50K outer membrane protein, and P. haemolytica leukotoxin. Thus, any of these proteins may be used alone or in combination with one or more of the other disclosed proteins in a vaccine composition according to the present invention.

In another embodiment of the present invention, the vaccine composition includes a pharmaceutically acceptable vehicle, an adjuvant and a subunit antigen composition comprising an immunogenic polypeptide comprising an immunogenic amino acid sequence of P. haemolytica leukotoxin, or an amino acid sequence substantially homologous and functionally equivalent thereto, and a saline extract of P. haemolytica.

Other embodiments of the present invention include isolated P. haemolytica fimbrial protein, plasmin receptor protein and 50K outer membrane protein.

Still other embodiments of the instant invention include DNA constructs comprising an expression cassette including (a) a DNA coding sequence for a polypeptide containing at least one epitope of a P. haemolytica protein selected from the group consisting of P. haemolytica fimbrial protein, P. haemolytica plasmin receptor protein, and P. haemolytica 50K outer membrane protein; and (b) control sequences that are operably linked to the coding sequence whereby the coding sequence can be transcribed and translated in a host cell.

Another embodiment of the invention is directed to the plasmid pAA352 (ATCC No. _______).

The present invention is also directed to host cells transformed with these constructs, as well as methods of making recombinant polypeptides useful in a P. haemolytica subunit vaccine.

In still other embodiments of the present invention, vaccination methods are provided for preventing or ameliorating respiratory disease in a ruminant.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Brief Description of the Figures

Figure 1 depicts the structure of the leukotoxin gene of P. haemolytica cloned in E. coli (Plasmid pAA114).

Figure 2 depicts the structure of Plasmid pAA101.

Figure 3 depicts the predicted amino acid sequence of the lktA::lacZ fusion protein from Plasmid pAA101. The portion representing the leukotoxin lktA protein is boxed.

Figure 4 depicts the structure of Plasmid pAA352 wherein tac is the hybrid trp::lac promoter from E. coli; bla represents the beta lactamase gene (ampicillin resistance); ori is the ColEl-based plasmid origin of replication; lktA is the Pasteurella haemolytica leukotoxin structural gene; and lacl is the E. coli lac operon repressor. The direction of transcription/translation of the leukotoxin gene is indicated by the arrow. The size of each component is not drawn to scale.

Figure 5 shows the nucleotide sequence and predicted amino acid sequence of leukotoxin 352 (LKT 352) from plasmid pAA352. Both the structural gene for LKT 352 and the sequences of the flanking vector regions are shown.

Figure 6 represents el ctron micrographs of P. haemolytica strain B122 fimbrial fractions. The bar

equals 100 nm. 6A shows PH-K fimbriae present in a crude shear fraction. 6B shows PH-K fimbriae following CsCl ultracentrifugation (density = 1.32 g/ml). 6C shows PH-K fimbriae following CsCl ultracentrifugation and incubation with 5M urea.

Figure 7 depicts the chromatofocusing pattern of CsCl purified fimbriae.

Figure 8 shows the temperature response in calves challenged with bovine herpes virus-1 and *P. haemolytica* after vaccination with *P. haemolytica* fimbriae as described in Example II, vaccination trial 1.

Figure 9 summarizes the clinical scores obtained in Example II, vaccination trial 1.

Figure 10 shows the mean ELISA titers obtained in Example II, vaccination trial 2.

Figure 11 depicts the mean clinical scores obtained in Example II, vaccination trial 2.

Figure 12 shows the temperature response in calves from Example II, vaccination trial 2.

Figure 13 depicts the lung scores obtained in calves from Example II, vaccination trial 2.

Figure 14 shows the outer membrane profile of *P. haemolytica* A1. The arrow indicates the position of the plasmin receptor protein.

Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, virology, recombinant DNA technology, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. *See*, *e.g.*, Maniatis, Fritsch & Sambrook, *Molecular Cloning: A Laboratory Manual* (1982); *DNA Cloning*, Vols. I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds.

1984); Animal Cell Culture (R.K. Freshney ed. 1986); Immobiliz d Cells and Enzymes (IRL press, 1986); B. Perbal, A Practical Guide to Molecular Cloning (1984); the series, Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.); and Handbook of Experimental Immunology, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., 1986, Blackwell Scientific Publications).

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

An "antigen" refers to a molecule containing one or more epitopes that will stimulate a host's immune system to make a humoral and/or cellular antigen-specific response. The term is also used interchangeably with "immunogen."

A "hapten" is a molecule containing one or more epitopes that does not stimulate a host's immune system to make a humoral or cellular response unless linked to a carrier.

The term "antigenic determinant" refers to the site on an antigen or hapten to which a specific antibody molecule binds. The term is also used interchangeably with "epitope" or "antigenic determinant site."

An "immunological response" to a composition or vaccine is the development in the host of a cellular and/or antibody-mediated immune response to the composition or vaccine of interest. Usually, such a response consists of the subject producing antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells directed specifically to an antigen or antigens included in the composition or vaccine of interest.

An "immunogenic polypeptide" or "immunogenic amino acid sequence" is a polypeptide or aminc acid

sequence, respectively, which elicits an immunological response in a subject to which it is administered.

By "subunit antigen composition" is meant a composition containing at least one immunogenic polypeptide, but not all antigens, derived from or homologous to an antigen from a pathogen of interest. Such a composition is substantially free of intact pathogen cells or particles, or the lysate of such cells or particles. Rather, a "subunit antigen composition" is prepared from at least partially purified (preferably substantially purified) immunogenic polypeptides from the pathogen, or recombinant analogs thereof. Thus, a subunit antigen composition can comprise the subunit antigen or antigens of interest substantially free of other antigens or polypeptides from the pathogen.

A "substantially enriched" vaccine composition is one where an antigen is derived from a cellular source having an increased concentration of the desired antigen or antigens with respect to the concentration of antigen or antigens found under normal growth conditions. A composition can be "substantially enriched" by adding additional amounts of one or more of the antigens to the composition, by altering growth conditions to increase production of the desired antigen or antigens, or by selectively fractionating a cell lysate to enhance the amount of the desired antigen or antigens.

The term "protein" is used herein to designate a naturally occurring polypeptide. The term "polypeptide" is used in its broadest sense, i.e., any polymer of amino acids (dipeptide or greater) linked through peptide bonds. Thus, the term "polypeptide" includes proteins, oligopeptides, protein fragments, analogs, muteins, fusion proteins and the like.

"Native" proteins or polypeptides refer to proteins or polypeptides recovered from a source occurring in nature. Thus, the term "native _P. haemolytica_ poly-

peptide" would include naturally occurring P. haemolytica proteins and fragments thereof. "Recombinant" polypeptides refer to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide. "Synthetic" polypeptides are those prepared by chemical synthesis.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; i.e., capable of replication under its own control.

A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "double-stranded DNA molecule" refers to the polymeric form of deoxyribonucleotides (bases adenine, guanine, thymine, or cytosine) in a double-stranded helix, both relaxed and supercoiled. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5′ to 3′ direction along the nontranscribed strand of DNA (i.e., the strand having the sequence homologous to the mRNA).

A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxy) terminus. A coding sequence can include, but is not limited to,

procaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3′ to the coding sequence.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3′ direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bound at the 3′ terminus by the translation start codon (ATG) of a coding sequence and extends upstream (5′ direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eucaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Procaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

DNA "control sequences" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell.

A coding sequence is "operably linked to" or "under the control of" control sequences in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an exogenous DNA sequence.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) to chromosomal DNA making up the genome of the cell. In procaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eucaryotic cells, a stably transformed cell is one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones comprised of a population of daughter cell containing the exogenous DNA.

A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth _in vitro_ for many generations.

Two DNA or polypeptide sequences are "substantially homologous" when at least about 80% (preferably at least about 90%, and most preferably at least about 95%) of the nucleotides or amino acids match over a defined length of the molecule. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. _See_, _e.g._, Maniatis et al., supra; _DNA Cloning_, vols I & II, supra; _Nucleic Acid Hybridization_, supra.

The term "functionally equivalent" intends that the amino acid sequence of the subject polypeptide is one that will elicit an immunological response, as defined

above, equivalent to a P. haemolytica immunogenic polypeptide.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. Thus, when the heterologous region encodes a bacterial gene, the gene will usually be flanked by DNA that does not flank the bacterial gene in the genome of the source bacteria. Another example of the heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Allelic variation or naturally occurring mutational events do not give rise to a heterologous region of DNA, as used herein.

A composition containing A is "substantially free of" B when at least about 85% by weight of the total of A + B in the composition is A. Preferably, A comprises at least about 90% by weight of the total of A + B in the composition, more preferably at least about 95%, or even 99% by weight.

The term "treatment" as used herein refers to either (i) the prevention of infection or reinfection (prophylaxis), or (ii) the reduction or elimination of symptoms or the disease of interest (therapy).

B. General Methods

Central to the instant invention is the discovery of several cell surface antigens of P. haemolytica including P. haemolytica fimbrial protein, P. haemolytica plasmin receptor protein, P. haemolytica 50K outer membrane protein and P. haemolytica leukotoxin. These proteins can be used, either alone or in combination, in a vaccine composition to protect animals against respiratory diseases such as pneumonia, including shipping fever pneumonia. Each of these proteins, or a mixture of

two or more of the proteins, can be further combined with saline extracted antigens, sodium salicylate capsular extracts, or antigens extracted by other methods known in the art, to produce a vaccine useful in protecting an animal against shipping fever or other respiratory disease.

P. haemolytica bears at least two types of fimbriae or pili, one thick and rigid and the other thin and flexible. Such fimbriae can be isolated from bacteria grown under routine conditions. For example, fimbriae can be isolated from P. haemolytica cultures grown on brain heart infusion agar at 37°C. Additionally, it may be possible to enhance fimbrial growth by subjecting various bacteria to elevated temperatures or increased iron levels. Doorn et al. (1987) Microbial Pathogenesis 3:87-95.

The thick and rigid fimbriae from P. haemolytica A1, referred to as "PH-K fimbriae," can be seen in a crude shear fraction as illustrated in Figure 4A. The fraction can be further purified by centrifugation through a CsCl step gradient, rendering fimbrial structures as seen in Figure 4B. The fimbriae thus isolated have a density of 1.32 and a molecular weight of approximately 35,000, as determined by SDS-polyacrylamide gel electrophoresis and immunoblotting with monoclonal antibodies raised against native fimbriae. The fimbriae are approximately 12 nm in diameter and vary in length from <100 nm to more than 500 nm. The isoelectric point of the purified fimbriae is 4.8.

The purified fimbrial protein includes the following amino acid sequence:

xxxxxxIle-Ala-Ala-Leu-Asn-Thr-Leu-Asn-Arg-Leu-Ser-Ala-Asn-Asn-Gly-Ala-S r-Gln-Lys-Asn-(Met-Phe).

where x is unknown.

The fimbrial protein can also be purified using standard immunoadsorption techniques well known in the

art. Additionally, the fimbrial protein can be produced via recombinant methods as described more fully below.

The protein has been used in vaccine trials as discussed in the experimental section and protects subjects from subsequent challenge with P. haemolytica.

Also of importance to the instant invention is the identification of a receptor protein on the surface of P. haemolytica able to bind both plasmin and plasminogen. Plasmin receptors have only recently been found in association with one other bacterium -- group A streptococcus. See Lottenberg et al., supra; Broseker et al., supra. Furthermore, unlike Pasteurella receptors, plasmin receptors of group A streptococcus are unable to bind the inactive zymogen, plasminogen.

These plasmin receptors can be detected by adding P. haemolytica to a substrate known to be degraded by plasmin. For example, fibrin plate assays, casein degradation assays using skim milk-agarose plates, as well as other assays, well known in the art, can be used for this purpose. The identity of the receptor protein can further be established by using standard techniques including separation using electrophoretic techniques, electroblotting the components to a nitrocellulose membrane and reacting the products with biotinylated plasmin or plasminogen. Identification procedures are described more fully in the experimental section. The receptor has a molecular weight of approximately 41,000 as determined by SDS-PAGE. The receptor protein can also be purified via immunoadsorption or produced by recombinant means as discussed below.

Also of importance is the discovery of a new outer membrane protein of P. haemolytica. This protein has a molecular weight of 50,000 as determined by SDS-PAGE. Immunologically-related proteins of the same molecular weight are produced by P. haemolytica serotypes 1, 2, 5, 6, 7, 8, 9 and 12. The protein can be purified

by elution from polyacrylamide gels of separated outer membrane components. This protein, alone or in combination with the proteins described above, is useful in protecting subjects from subsequent challenge with bovine herpes virus-1 followed by exposure to P. haemolytica.

Also useful in the vaccines of the present invention is P. haemolytica leukotoxin. Actively growing cells of P. haemolytica have been shown to secrete leukotoxin which can also be cloned, expressed and used either alone or in combination with one or more of the above antigens to immunize subjects against shipping fever. The nucleotide sequence coding for P. haemolytica A1 leukotoxin has been determined. See, e.g., Lo, R.Y.C. (1987) Infect. Immun. 55:1987-1996. Of interest is the fact that the P. haemolytica leukotoxin gene and the corresponding protein share extensive homology with Escherichia coli alpha hemolysin (50.3% of the amino acid residues are identical). Strathdee, C.A., and Lo, R.Y.C. (1987) Infect. Immun. 55:3233-3236. P. haemolytica leukotoxin can be produced using recombinant techniques and purified from, for example, bacterial cells. The leukotoxin can also be purified from native bacteria using immunoadsorbent chromatography. The molecular weight of the purified leukotoxin is approximately 95,000 and the isoelectric point is 6.3.

Saline extracts of P. haemolytica can also be combined with any of the above subunit antigens. These extracts are produced by extracting proteins in an 0.85% (w/v) sodium chloride solution. The extract can be further treated, i.e. with glass beads and agitation, or other methods known in the art, to remove cell surface proteins. The combination of such saline extracts with isolated or recombinantly produced leukotoxin affords potent protection against shipping fever.

Purification of the above antigens as described herein permits the sequencing of the same by any of the

various methods known to those skilled in the art. For example, the amino acid sequences of the subject proteins can be determined from the purified proteins by repetitive cycles of Edman degradation, followed by amino acid analysis by HPLC. Other methods of amino acid sequencing are also known in the art.

Once the amino acid sequences are determined, oligonucleotide probes which contain the codons for a portion of the determined amino acid sequences can be prepared and used to screen DNA libraries for genes encoding the subject proteins. The basic strategies for preparing oligonucleotide probes and DNA libraries, as well as their screening by nucleic acid hybridization, are well known to those of ordinary skill in the art. See, e.g., DNA Cloning: Vol. I, supra; Nucleic Acid Hybridization, supra; Oligonucleotide Synthesis, supra; T. Maniatis et al., supra.

First, a DNA library is prepared. The library can consist of a genomic DNA library from *P. haemolytica*. Once the library is constructed, oligonucleotides to probe the library are prepared and used to isolate the gene encoding the desired protein. The oligonucleotides are synthesized by any appropriate method. The particular nucleotide sequences selected are chosen so as to correspond to the codons encoding a known amino acid sequence from the desired *P. haemolytica* protein. Since the genetic code is degenerate, it will often be necessary to synthesize several oligonucleotides to cover all, or a reasonable number, of the possible nucleotide sequences which encode a particular region of the protein. Thus, it is generally preferred in selecting a region upon which to base the probes, that the region not contain amino acids whose codons are highly degenerate. In certain circumstances, one of skill in the art may find it desirable to prepare probes that are fairly long, and/or encompass regions of the amino acid sequence which would

have a high degree of redundancy in corresponding nucleic acid sequences, particularly if this lengthy and/or redundant region is highly characteristic of the protein of interest. It may also be desirable to use two probes (or sets of probes), each to different regions of the gene, in a single hybridization experiment. Automated oligonucleotide synthesis has made the preparation of large families of probes relatively straightforward. While the exact length of the probe employed is not critical, generally it is recognized in the art that probes from about 14 to about 20 base pairs are usually effective. Longer probes of about 25 to about 60 base pairs are also used.

The selected oligonucleotide probes are labeled with a marker, such as a radionucleotide or biotin using standard procedures. The labeled set of probes is then used in the screening step, which consists of allowing the single-stranded probe to hybridize to isolated ssDNA from the library, according to standard techniques. Either stringent or permissive hybridization conditions could be appropriate, depending upon several factors, such as the length of the probe and whether the probe is derived from the same species as the library, or an evolutionarily close or distant species. The selection of the appropriate conditions is within the skill of the art. See generally, Nucleic Acid hybridization, supra. The basic requirement is that hybridization conditions be of sufficient stringency so that selective hybridization occurs; i.e., hybridization is due to a sufficient degree of nucleic acid homology (e.g., at least about 75%), as opposed to nonspecific binding. Once a clone from the screened library has been identified by positive hybridization, it can be confirmed by restriction enzyme analysis and DNA sequencing that the particular library insert contains a gene for th desired protein.

Alternatively, DNA sequences encoding the proteins of interest can be pr pared synthetically rather than cloned. The DNA sequence can be designed with the appropriate codons for the particular Pasteurella amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge (1981), Nature 292:756; Nambair et al. (1984) Science 223:1299; Jay et al. (1984) J. Biol. Chem. 259:6311.

Once a coding sequence for the desired protein has been prepared or isolated, it can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and host cells which they can transform include the bacteriophage lambda (E. coli), pBR322 (E. coli), pACYC177 (E. coli), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-E. coli gram-negative bacteria), pHV14 (E. coli and Bacillus subtilis), pBD9 (Bacillus), pIJ61 (Streptomyces), pUC6 (Streptomyces), YIp5 (Saccharomyces), YCp19 (Saccharomyces) and bovine papilloma virus (mammalian cells). See, generally, DNA Cloning: Vols. I & II, supra; T. Maniatis et al., supra; B. Perbal, supra.

The coding sequence for the Pasteurella protein of interest can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence encoding the protein is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding sequence may or may not contain a signal peptide or leader sequ nce. The full length P.

haemolytica proteins of the present invention can be express d using, for example, native P. haemolytica promoter or the protein A gene (spa) promoter and signal sequence. Truncated versions of the genes of interest can be fusions with the B-galactosidase gene (lacZ) as well as genes coding for galactokinase (galK) and interleukin-2 (bovine). Leader sequences can be removed by the bacterial host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397.

In addition to control sequences, it may be desirable to add regulatory sequences which allow for regulation of the expression of the bacterial antigen sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences. The subject proteins can also be expressed in the form of a fusion protein, wherein a heterologous amino acid sequence is expressed at the N-terminal. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437.

An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the sequences encoding the particular antigen of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; i.e., to

maintain th reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

In some cases, it may be desirable to add sequences which cause the secretion of the polypeptide from the host organism, with subsequent cleavage of the secretory signal. It may also be desirable to produce mutants or analogs of the antigen of interest. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the antigen, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. See, e.g., T. Maniatis et al., supra; DNA Cloning, Vols. I and II, supra; Nucleic Acid Hybridization, supra.

A number of procaryotic expression vectors are known in the art. See, e.g., U.S. Patent Nos. 4,440,859; 4,436,815; 4,431,740; 4,431,739; 4,428,941; 4,425,437; 4,418,149; 4,411,994; 4,366,246; 4,342,832; see also U.K. Patent Applications GB 2,121,054; GB 2,008,123; GB 2,007,675; and European Patent Application 103,395. Yeast expression vectors are also known in the art. See, e.g., U.S. Patent Nos. 4,446,235; 4,443,539; 4,430,428; see also European Patent Applications 103,409; 100,561; 96,491.

Depending on the expression system and host selected, the proteins of the present invention are produced by growing host cells transformed by an expression v ctor described above under conditions whereby the prot in of interest is expressed. The particular Past urella protein is then isolated from the host cells and purified. If the expression system secretes the

protein into growth media, the protein can be purified directly from the media. If the protein is not secreted, it is isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

An alternative method to identify antigens of the present invention is by constructing gene libraries, using the resulting clones to transform _E. coli_ and pooling and screening individual colonies using polyclonal serum or monoclonal antibodies to the desired antigen.

The antigens of the present invention can also be isolated from _P. haemolytica_ cultures using standard protein purification procedures well known in the art. _See, e.g., Protein Purification Principles and Practice_ 2d edition (Robert K. Scopes ed. 1987). Such techniques include gel filtration chromatography, ion exchange chromatography, affinity chromatography, immunoadsorbent chromatography, polyacrylamide gel electrophoresis and other electrophoretic techniques, centrifugation, dialysis, and precipitation.

The antigens of the present invention may also be produced by chemical synthesis such as solid phase peptide synthesis, using known amino acid sequences or amino acid sequences derived from the DNA sequence of the gene of interest. Such methods are known to those skilled in the art. Chemical synthesis of peptides may be preferable if a small fragment of the antigen in question is capable of raising an immunological response in the subject of interest.

The proteins of the present invention or their fragments can be used to produce antibodies, both polyclonal and monoclonal. If polyclonal antibodies are desired, a selected mammal, (e.g., mouse, rabbit, goat, horse, etc.) is immunized with an antigen of th present invention, or its fragment, or a mutated antigen. Serum from the immunized animal is collected and treated accord-

ing to known procedures. If serum containing polyclonal antibodies is used, the polyclonal antibodies can be purified by immunoaffinity chromatography, using known procedures.

Monoclonal antibodies to the proteins of the present invention, and to the fragments thereof, can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier et al., Hybridoma Techniques (1980); Hammerling et al., Monoclonal Antibodies and T-cell Hybridomas (1981); Kennett et al., Monoclonal Antibodies (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,452,570; 4,466,917; 4,472,500, 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against the antigen of interest, or fragment thereof, can be screened for various properties; i.e., for isotype, epitope, affinity, etc. Monoclonal antibodies are useful in purification, using immunoaffinity techniques, of the antigens which they are directed against.

Animals can be immunized with the compositions of the present invention by administration of the protein of interest, or a fragment thereof, or an analog thereof. If the fragment or analog is used, it will include the amino acid sequence of the epitope which interacts with the immune system to immunize the animal to that and structurally similar epitopes.

Fragments used to immunize a subject contain at least 6-30 amino acids which form the sequence of the desired protein, and include the particular epitope. Smaller fragments encompassing the epitope may be inserted into larger peptides or polypeptid s, such that the

regions flanking the epitope are not those that are encod d within the naturally occurring genes. The techniques for the synthesis of these peptides or polypeptides are apparent to one of average skill in the art. For example, the genetic sequence encoding a particular antigen may be isolated via cloning, and that sequence altered at sites other than that encoding the particular epitope. This alteration may be accomplished by site specific mutation, or by deletions, or by insertions. Alternatively, an oligonucleotide sequence encoding the epitope may be inserted into or attached to another sequence which encodes a different peptide or polypeptide. A recombinant sequence is then inserted into an expression vector which is compatible with the host to be transformed, and the expression system used to synthesize the desired peptide which includes the particular epitope. The techniques by which this may be accomplished are known to those of skill in the art. See, e.g., T. Maniatis et al., supra; DNA Cloning, Vols. I and II, supra; and Nucleic Acid Hybridization, supra. Alternatively, an oligopeptide may be synthesized by solid phase synthesis which includes the particular epitope, but which adds flanking amino acids to it which are not in the sequence of the naturally occurring antigen.

Prior to immunization, it may be desirable to increase the immunogenicity of the particular Pasteurella protein, or an analog of the protein, or particularly fragments of the protein. This can be accomplished in any one of several ways known to those of skill in the art. For example, the antigenic peptide may be administered linked to a carrier. For example, a fragment may be conjugated with a macromolecular carrier. Suitable carriers are typically large, slowly metabolized macromolecules such as: proteins; polysaccharides, such as sepharose, agarose, cellulose, cellulose beads and the like; polymeric amino acids such as polyglutamic acid,

polylysine, and the like; amino acid copolymers; and inactive virus particles. Especially useful protein substrates are serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, and other proteins well known to those skilled in the art.

The protein substrates may be used in their native form or their functional group content may be modified by, for example, succinylation of lysine residues or reaction with Cys-thiolactone. A sulfhydryl group may also be incorporated into the carrier (or antigen) by, for example, reaction of amino functions with 2-iminothiolane or the N-hydroxysuccinimide ester of 3-(4-dithiopyridyl propionate. Suitable carriers may also be modified to incorporate spacer arms (such as hexamethylene diamine or other bifunctional molecules of similar size) for attachment of peptides.

Other suitable carriers for the proteins/fragments/analogs of the present invention include VP6 polypeptides of rotaviruses, or functional fragments thereof, as disclosed in commonly owned U.S. Patent Application Serial Number 092,120, incorporated herein by reference. Also useful is a fusion product of a viral protein and the epitope of interest made by methods disclosed in U.S. Patent No. 4,722,840. Still other suitable carriers include cells, such as lymphocytes, since presentation in this form mimics the natural mode of presentation in the subject, which gives rise to the immunized state. Alternatively, the antigens of the present invention, or an antigenic fragment thereof, or analog thereof, may be coupled to erythrocytes, preferably the subject's own erythrocytes. Methods of coupling peptides to proteins or cells are known to those of skill in the art.

It is also possible to immunize a subject with a protein of the present invention, or a protective fragment thereof, or an analog thereof, which is administered

alone, or mixed with a pharmaceutically acceptable vehicle or excipient. Typically, vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation may also be emulsified or the active ingredient encapsulated in liposome vehicles. The active immunogenic ingredient is often mixed with vehicles containing excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable vehicles are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vehicle may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccine. Adjuvants may include for example, muramyl dipeptides, avridine, aluminum hydroxide, oils, saponins and other substances known in the art. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in the art. See, e.g., Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania, 15th edition, 1975. The composition or formulation to be administered will, in any event, contain a quantity of the protein adequate to achieve the desired immunized state in the individual being treated.

Additional vaccine formulations which are suitable for other modes of administration include suppositories and, in some cases, aerosol, intranasal and oral formulations. For suppositories, the vehicle composition will include traditional binders and carriers, such as, polyalkaline glycols, or triglycerides. Such suppositories may be formed from mixtures containing the active ingr dient in the range of about 0.5% to about 10% (w/w), preferably about 1% to about 2%. Oral vehicles include such normally employed excipients as, for example,

pharmaceutical grades of mannitol, lactose, starch, magnesium, stearate, sodium saccharin cellulose, magnesium carbonate, and the like. These oral vaccine compositions may be taken in the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders, and contain from about 10% to about 95% of the active ingredient, preferably about 25% to about 70%.

Intranasal formulations will usually include vehicles that neither cause irritation to the nasal mucosa nor significantly disturb ciliary function. Diluents such as water, aqueous saline or other known substances can be employed with the subject invention. The nasal formulations may also contain preservatives such as, but not limited to, chlorobutanol and benzalkonium chloride. A surfactant may be present to enhance absorption of the subject proteins by the nasal mucosa.

Furthermore, the P. haemolytica antigens (or complexes thereof) may be formulated into vaccine compositions in either neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the active polypeptides) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

To immunize a subject, the polypeptide of interest, or an immunologically active fragment thereof, is administered parenterally, usually by intramuscular injection in an appropriate vehicle. Other modes of administration, however, such as subcutaneous, intravenous injection and intranasal d livery, are also acceptable. Injectable vaccine formulations will contain an effective

amount of the active ingredient in a vehicle, the exact amount being readily d termined by one skilled in the art. The active ingredient may typically range from about 1% to about 95% (w/w) of the composition, or even higher or lower if appropriate. The quantity to be administered depends on the animal to be treated, the capacity of the animal's immune system to synthesize antibodies, and the degree of protection desired. It has been found that in the present vaccine formulations, 50 ug of active ingredient per ml of injected solution is adequate to raise an immunological response when a dose of 1 to 5 ml per animal is administered. Other effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves. The subject is immunized by administration of the particular antigen or fragment thereof, or analog thereof, in at least one dose, and preferably two doses. Moreover, the animal may be administered as many doses as is required to maintain a state of immunity to pneumonia.

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Deposits of Strains Useful in Practicing the Invention

A deposit of biologically pure cultures of the following strains was made with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland. The accession number indicated was assigned after successful viability testing, and the requisite fees were paid. Access to said cultures will be available during pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 USC 122. All restriction on availability of said cultures to the public will be irrevocably removed upon the granting of a patent based upon the application.

Moreover, the designated deposits will be maintained for a period of thirty (30) y ars from the date of deposit, or for five (5) years after the last request for the deposit; or for the enforceable life of the U.S. patent, whichever is longer. Should a culture become nonviable or be inadvertently destroyed, or, in the case of plasmid-containing strains, lose its plasmid, it will be replaced with a viable culture(s) of the same taxonomic description.

Strain	Deposit Date	ATCC No.
P. haemolytica serotype 1 B122	February 1, 1989	53863
pAA213 in E. coli JM105	February 1, 1989	67882
pAA101 in E. coli JM105	February 1, 1989	67883
pAA352 in E. coli W1485	March 30, 1990	______

C. Experimental

Materials and Methods

Enzymes were purchased from commercial sources, and used according to the manufacturers' directions. Radionucleotides and nitrocellulose filters were also purchased from commercial sources.

In the cloning of DNA fragments, except where noted, all DNA manipulations were done according to standard procedures. See T. Maniatis et al., supra. Restriction enzymes, T_4 DNA ligase, E. coli, DNA polymerase I, Klenow fragment, and other biological reagents were purchased from commercial suppliers and used according to the manufacturers' directions. Double-stranded DNA fragments were separated on agarose gels.

cDNA and genomic libraries were prepared by standard techniques in pUC13 and the bacteriophage lambda gt11, respectively. See DNA CLONING: Vols I and II, supra.

P. haemolytica biotype A, serotype 1 ("A1") strain B122 was isolated from the lung of a calf which died of pneumonic pasteurellosis and was stored at -70°C in defibrinated blood. Routine propagation was carried out on blood agar plates or in brain heart infusion broth (Difco Laboratories, Detroit, MI) supplemented with 5% (v/v) horse serum (Gibco Canada Ltd., Burlington, Canada). All cultures were incubated at 37°C.

Example 1

The protective capacity of P. haemolytica leukotoxin and 50K outer membrane protein were tested by administering the recombinant and/or authentic products listed in Table 1 to calves.

Table 1

Proteins Administered To Calves In Example 1

(1) Recombinant 50K outer membrane protein.
(2) Control group -- avridine (adjuvant) only.
(3) Authentic leukotoxin.
(4) Recombinant leukotoxin:B-galactosidase (from pAA101 described below).
(5) Recombinant leukotoxin (from pAA352 described below).
(6) Recombinant 50K outer membrane protein plus authentic leukotoxin.
(7) Recombinant outer membrane protein plus recombinant leukotoxin.
(8) Saline-extract of P. haemolytica.
(9) Saline-extract plus authentic leukotoxin.
(10) Saline-extract plus recombinant leukotoxin (from pAA352).

The products from Table 1 wer made as follows.

Purification of P. haemolytica Authentic Leukotoxin

P. haemolytica A1 was grown to mid-log phase in Brain Heart Infusion broth (Difco) at 37°C. Cells were harvested by centrifuging at 9,000 rpm for 20 minutes using a Sorval GSA rotor. The supernatant was transferred to a fresh flask and ammonium sulfate added to 70% saturation. The mixture was stirred overnight in the cold. The precipitate was collected by centrifugation as above and the pellet was dissolved in 10 ml sterile water per liter of the original culture. The dissolved pellet was desalted by passage through Sephadex G-25. The collected sample was subjected to preparative isoelectric focusing using a rotofor apparatus (Biorad Labs) over a pH gradient of 5 to 7. Fractions over a range of pH 6-7 were pooled and NaCl added to 1.0 M. The sample was passed through a Sephadex G-25 column. The purified leukotoxin was loaded on a 12.5% SDS-polyacrylamide gel and the sample contained one major protein band corresponding to a molecular weight of 95,000.

1. Production of P. haemolytica Recombinant Leukotoxin from pAA101

To produce recombinant leukotoxin, gene libraries of *P. haemolytica* A1 (strain B122) were constructed using standard techniques. See Lo et al., Infect. Immun., supra; DNA CLONING: Vols. I and II, supra; and T. MANIATIS et al., supra. A genomic library was constructed in the plasmid vector pUC13 and a DNA library constructed in the bacteriophage lambda gt11. The resulting clones were used to transform *E. coli* and individual colonies were pooled and screened for reaction with serum from a calf which had survived a *P. haemolytica* infection and that had been boosted with a concentrated culture supernatant of *P. haemolytica* to increase anti-leukotoxin antibody levels. Positive colonies were screened for their ability to produce leukotoxin by incubating cell lysates

with bovine neutrophils and subsequently measuring release of lactate dehydrogenase from the latter.

Several positive colonies were identified and these recombinants were analyzed by restriction endonuclease mapping. One clone appeared to be identical to a leukotoxin gene cloned previously. See Lo et al., Infect. Immun, supra. To confirm this, smaller fragments were recloned and the restriction maps compared. It was determined that approximately 4 kilobase pairs of DNA had been cloned. Progressively larger clones were isolated by carrying out a chromosome walk (5′ to 3′ direction) in order to isolate full length recombinants which were approximately 8 kb in length. The final construct was termed pAA114. This construct contained the entire leukotoxin gene sequence. The structure of this plasmid is shown in Figure 1.

Both full length and truncated versions of the leukotoxin gene were expressed. The truncated forms were fusions with B-galactosidase (lacZ). The full length versions were expressed using the native <u>P. haemolytica</u> promoter or the protein A gene (spa) promoter and signal sequence. Clones were constructed as follows.

Plasmids pLTX1.1 and pLTX3.2 were isolated from <u>P. haemolytica</u> genomic DNA as purified restriction fragments (1.0 kb and 2.1 kb, respectively) from an EcoRV Pstl double digest. These fragments were cloned into pTZ18R digested with HincII Pstl. The vector was used to transform <u>E. coli</u> strain JM105. Transformed cells were identified by plating on media containing ampicillin plus Xgal and IPTG. Blue colonies indicated the presence of a functional lacZ gene. The DNA from these colonies was analyzed by restriction endonuclease digestion and found to contain the 5′ end of the leukotoxin gene (lktC + lktA). This plasmid was termed pLTX3P.1.

Plasmid pLTX3P.1 was mutagenized in vitro with hydroxylamine, transformed into JM105 and plated on a

growth medium containing ampicillin plus a reduced concentration of Xgal. In this way clones expressing increased quantities of the lktA:lacZ product would be dark blue whereas those containing an unmodified gene would be white or light blue. The clones from the dark blue colonies were termed pAA134.

A leukotoxin EcoRV/Pst1 5'-fragment (from pLTX3P.1) was subcloned into pBR325 digested with EcoR1/Pst1 containing the native leukotoxin promoter (from pLTX3P.1), plus a promoterless full length lacZ gene from plasmid pMC1871 (Pst1 fragment). The plasmid was used to transform E. coli JM105 and blue colonies were isolated from Xgal agar. This plasmid was termed pAA101 and is illustrated in Figure 2. The predicted amino acid sequence of the fusion protein is shown in Figure 3.

2. Production of P. haemolytica Recombinant Leukotoxin from pAA352

A second version of recombinant P. haemolytica leukotoxin was expressed. This leukotoxin was termed "leukotoxin 352" or "LKT 352". In order to produce this leukotoxin, the following gene construct was prepared from pAA114 described above.

lktA, a MaeI restriction endonuclease fragment which contained the entire gene was treated with the Klenow fragment of DNA polymerase I plus nucleotide triphosphates and ligated into the SmaI site of the cloning vector pUC13. This plasmid was named pAA179. From this, two expression constructs were made in the ptac-based vector pGH432: lacI digested with SmaI. One, pAA342, consisted of the 5'-AhaIII fragment of the lktA gene while the other, pAA345, contained the entire MaeI fragment described above. The clone pAA342 expressed a truncated leukotoxin peptide at high levels while pAA345 expressed full length leukotoxin at very low levels. Therefore, the 3' end of the lktA gene (StyI BamHI

fragment from pAA345) was ligated to StyI BamHI-digested pAA342, yielding the plasmid pAA352. Th structure of this plasmid is shown in Figure 4.

The nucleotide sequence of the leukotoxin expressed by plasmid pAA352 (LKT 352 or new leukotoxin) is shown in Figure 5. The peptide encoded by this sequence is 931 amino acids in length and is 98% homologous to authentic leukotoxin. This recombinant leukotoxin migrates, on polyacrylamide gels, to a position identical to authentic leukotoxin.

3.a. Purification of Recombinant Leukotoxin from Example 1.1

Two liters of *E. coli* JM105/pAA101 were grown in broth to mid-exponential growth phase and the cells harvested by centrifugation. The pellet was resuspended in 50 ml of TEP buffer (100mM Tris-HCl, pH 7.4, 10mM EDTA, 1mM phenyl methyl sulfonyl fluoride), immediately frozen at -70°C and held overnight. The cells were then thawed and sonicated for a total of 4 minutes (30 second bursts, 200 W) and the cell debris removed by centrifugation at 10,000 rpm in a Sorvall SS-34 rotor. The supernatant was mixed with three volumes of saturated ammonium sulfate and stirred at 4°C for 60 minutes. This slurry was stored at 4°C overnight then centrifuged as above. The pellet obtained from *E. coli* JM105/pAA101 cells was dissolved in 10 ml of TEP buffer and diluted to 20 ml with TBSN (10mM Tris-HCl, pH 8, 500 mM NaCl, 0.2% NP-40). This solution was passed through an affinity column containing a monoclonal antibody to B-galactosidase ("Protosorb" from Promega Biotech). The column was washed once with 20 ml TBSN. The fusion protein was eluted with 5.0 ml of 0.1 M $NaHCO_3/Na_2CO_3$, pH 10.8, and stored at 4°C.

3.b. Purification of Recombinant Leukotoxin (LKT 352) from Example 1.2

Recombinant LKT 352 was purified using the following procedure. Five to ten colonies of *E. coli* W1485/pAA352 (ATCC no. _____) were inoculated into 10 ml of TB broth supplemented with 100 micrograms/ml of ampicillin and incubated at 37°C for 6 hours on a G10 shaker, 220 rpm. Four ml of this culture was diluted into each of two baffled Fernbach flasks containing 400 ml of TB broth + ampicillin and incubated overnight as described above. Cells were harvested by centrifugation for 10 minutes at 4,000 rpm in polypropylene bottles, 500 ml volume, using a Sorvall GS3 rotor. The pellet was resuspended in an equal volume of TB broth containing ampicillin which had been prewarmed to 37°C (i.e., 2 x 400 ml), and the cells were incubated for 2 hours as described above.

3.2 ml of isopropyl-B,D-thiogalactopyranoside (IPTG, Gibco/BRL), 500 mM in water (final concentration = 4 mM), was added to each culture in order to induce synthesis of recombinant leukotoxin. Cultures were incubated for two hours. Cells were harvested by centrifugation as described above, resuspended in 30 ml of 50 mM Tris-hydrochloride, 25% (w/v) sucrose, pH 8.0, and frozen at -70°C. The frozen cells were thawed at room temperature after 60 minutes at -70°C, and 5 ml of lysozyme (Sigma, 20 mg/ml in 250 mM Tris-HCl, pH 8.0) was added. The mixture was vortexed at high speed for 10 seconds and then placed on ice for 15 minutes. The cells were then added to 500 ml of lysis buffer in a 1000 ml beaker and mixed by stirring with a 2 ml pipette. The beaker containing the lysed cell suspension was placed on ice and sonicated for a total of 2.5 minutes (5-30 second bursts with 1 minute cooling between each) with a Braun sonicator, large probe, set at 100 watts power. Equal volumes of the solution were placed in Teflon SS34

centrifuge tubes and centrifuged for 20 minutes at 10,000 rpm in a Sorvall SS34 rotor. The pellets were resuspended in a total of 100 ml of sterile double distilled water by vortexing at high speed, and the centrifugation step repeated. Supernatants were discarded and the pellets combined in 20 ml of 10 mM Tris-HCl, 150 mM NaCl, pH 8.0 (Tris-buffered saline) and the suspension frozen overnight at -20°C.

The recombinant leukotoxin suspension was thawed at room temperature and added to 100 ml of 8 M Guanidine HCl (Sigma) in Tris-buffered saline and mixed vigorously. A magnetic stir bar was placed in the bottle and the solubilized sample was mixed at room temperature for 30 minutes. The solution was transferred to a 2000 ml Ehrlenmyer flask and 1200 ml of Tris-buffered saline was quickly added. This mixture was stirred at room temperature for an additional 2 hours. 500 ml aliquots were placed in dialysis bags (Spectrum, 63.7 mm diameter, 6,000-8,000 MW cutoff, #132670, from Fisher scientific) and these were placed in 4,000 ml beakers containing 3,500 ml of Tris-buffered saline + 0.5 M Guanidine HCl. The beakers were placed in a 4°C room on a magnetic stirrer overnight after which dialysis buffer was replaced with Tris-buffered saline + 0.1 M Guanidine HCl and dialysis continued for 12 hours. The buffer was then replaced with Tris-buffered saline + 0.05 M Guanidine HCl and dialysis continued overnight. The buffer was replaced with Tris-buffered saline (no guanidine), and dialysis continued for 12 hours. This was repeated three more times. The final leukotoxin solution was poured into a 2000 ml plastic roller bottle (Corning) and 13 ml of 100 mM PMSF (in ethanol) was added to inhibit protease activity. The solution was stored at -20°C in 100 ml aliquots.

Purification of P. haemolytica Authentic 50K Outer Membrane Protein

Outer membrane preparations were electrophoresed on polyacrylamide gels after which the 50K outer membrane protein band was cut out as a single gel slice. Gel slices were crushed and suspended in 3 ml of a buffer consisting of 0.1% SDS, 0.05 M Tris-HCl (pH 7.9), 0.1 mM EDTA, 5 mM dithiothreitol, 0.2 M NaCl. This mixture was shaken for 2 hr at 37°C, after which the buffer without gel fragments was removed and dialyzed overnight against 1 liter of 10 mM ammonium bicarbonate. The dialyzed preparation was then lyophilized and the protein resuspended in phosphate-buffered saline prior to use.

Production of P. haemolytica Recombinant 50K Outer Membrane Protein

1. Cloning the 50K Protein Gene

The purified authentic 50K protein (50 ug) was mixed with Freund's incomplete adjuvant and injected intramuscularly into a New Zealand white rabbit. This immunization was repeated 14 days and 28 days later and the rabbit was bled 1 week after the last injection. The serum so derived contained high levels of antibodies against the 50K outer membrane protein. This antisera was used to immunologically screen a *P. haemolytica* gene library for recombinant clones expressing the 50K protein.

The lambda gt11 *P. haemolytica* gene library was constructed as described above for *P. haemolytica* leukotoxin. Plating of the library and the use of rabbit anti-50K antisera to select recombinant clones was done using established techniques (Maniatis, et al.; French et al. (1986) Anal. Biochem. 156:417-423). Plaques reacting with the anti-50K antisera were purified and propagated on *E. coli* strain Y1090 and transduced into *E. coli* strain Y1089 for high l vel expression of the 50K protein gene

and subsequent purification of recombinant 50K B-galactosidase fusion protein.

2. Expression of the 50K Protein Gene

The recombinant 50K protein was expressed directly in *E. coli* Y1089 as a fusion product with B-galactosidase. *E. coli* Y1089 containing the recombinant lambda gt11 clone was grown at 32°C until an OD_{600} of 0.5 was reached. The culture was then shifted to 44°C and grown for 20 min. Isopropyl-thiogalactopyranoside was then added to 10 mM (final concentration) and the culture was incubated at 37°C for 1 hr. The cells were then harvested, broken by sonication, and the cell debris removed by centrifugation at 10,000 x g for 15 min. The supernatant containing the 50K B-galactosidase fusion protein was then subjected to ammonium sulfate precipitation as described above (see "Purification of Recombinant Leukotoxin").

3. Purification of Recombinant 50K Protein

The recombinant 50K protein was purified using the same method used to purify recombinant leukotoxin with the exception that the affinity column consisted of p-aminophenyl-B-D-thiogalactopyranoside bound to agarose beads (purchased from Sigma). The dissolved pellet from the ammonium sulfate precipitation was applied to a 5 ml affinity column which was then washed with a buffer consisting of 10 mM Tris-HCl (pH 7.6), 250 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA and 0.1% Triton X-100 to remove unbound protein. The bound protein was subsequently eluted with 5 ml of 100 mM sodium borate (pH 10.0). The eluted protein was then dialysed overnight against 1 l of phosphate buffered saline prior to use.

Preparation of P. haemolytica Saline Extract For Use in Vaccination Trial A

A one liter culture of P. haemolytica A1 (strain B122) was prepared in Brain Heart Infusion Broth (Difco) and the cells were harvested by centrifugation at 9,000 rpm for 20 minutes with a Sorvall GSA rotor. The pellet was washed once with 200 ml of 0.85% sodium chloride (w/v) which had been preheated to 65°C and and resuspended in 30 ml of the saline solution. The suspension was heated to 65°C for 20 minutes with continuous stirring and the bacteria removed by centrifugation. The supernatant was decanted and stored at 4°C.

Preparation of P. haemolytica Saline Extract for Use in Vaccination Trial D

A saline extract was made as above with the following modifications. Cells were harvested by centrifugation at 5,000 rpm for 10 minutes with a Sorvall GS3 rotor. After washing, the pellet was resuspended in 100 ml of the sodium chloride solution which had been preheated to 65°C. The suspension was placed in a large flask (preheated to 65°C), the bottom of which was covered with glass beads. The flask with cells was agitated vigorously in a New Brunswick G25 shaker (250-300 rpm) at 65°C for one hour. The sample was then centrifuged for 20 minutes at 10,000 rpm in a Sorvall SS34 rotor. The supernatant was carefully decanted into a sterile bottle. Phenylmethyl sulfonylfluoride was added to a final concentration of 0.1 mM and stored at 20°C.

Preparation of the Vaccine Compositions

Each dose of vaccine compositions 1-4 and 6-9, listed in Table 1 above, were prepared by mixing 1.0 ml of the antigen listed (100ug) 0.1 M PBS, pH 7.2, with an equal volume of freshly prepared avridine. Groups of six calves were vaccinated intramuscularly and boosted three

weeks later with the same vaccine composition. 10 days after boosting, the calves were exposed to bovine herpes virus-1 followed by exposure to P. haemolytica A-1 strain B122 four days later. Calves were monitored for clinical signs of disease, temperature and weight loss. The results of this trial (Vaccination Trial A) are shown in Table 2.

Table 2

Results of Vaccination Trial A of Example I

Group	Antigen	Mortality (# of calves dead/6 calves)	Presence of Pasteurellosis Symptoms (+)	Mean Clinical Score per Day
1	Recombinant 50K Protein	3	+	7.8
2	Control (avridine only)	5	+	10.7
3	Authentic Leukotoxin	1	+	4.0
4	Recombinant Leukotoxin	0	-	1.2
5	Recombinant 50K plus Authentic Leukotoxin	1	+	2.6
6	Recombinant 50K plus Recombinant Leukotoxin	3	+	6.3
7	Saline Extract	1	+	2.6
8	Saline Extract plus Authentic Leukotoxin	0	-	1.1

As illustrated in Table 2, groups 4 and 8 were completely protected while groups 3 and 5 were significantly protected. The control group, group 2, had the highest mortality rate. These results indicate that the recombinant leukotoxin:B-galactosidase fusion protein, as well as authentic leukotoxin, are effective immunogens for the prevention of bovine pneumonic pasteurellosis. Additionally, use of the 50K protein in combination with leukotoxin provided enhanced protection in comparison to the control group. It is possible that the protection afforded by the saline extract is at least partially due to the presence of leukotoxin.

A second vaccination trial (Vaccination Trial B) was carried out using the purified recombinant leukotoxin fusion protein described above. This protein was mixed with emulsigen (25% v/v) and calves vaccinated according to the groups listed in Table 3. The calves were boosted after 3 weeks and finally challenged with bovine herpes virus/P. haemolytica as described above. The results of this trial can be seen in Table 3.

Table 3

Results of Vaccination Trial B of Example 1

Group	Mortality
1. Emulsigen only	8/9
2. Emulsigen + 100 ug antigen	4/10
3. Emulsigen + 50 ug antigen	4/6
4. Emulsigen + 25 ug antigen	5/6
5. Emulsigen + 12.5 ug antigen	5/6

As can be seen, Groups 2 and 3, administered emulsigen plus 100 ug and 50 ug of antigen, r spectively, demonstrated a lower mortality rate than the control group. It should be noted that this experiment was done with a less than optimum adjuvant, possibly accounting for

the higher mortality rates over those seen in vaccination trial A.

The immunogenicity of recombinantly produced LKT 352, prepared as described above, was tested in a third vaccination trial (Vaccination Trial C) as follows. Twelve beef-type calves were randomized into two groups of six. The control group was vaccinated with placebo comprised of sterile saline combined with adjuvant. The second group was vaccinated with 100 ug of LKT 352 in adjuvant. Two injections were given intramuscularly 21 days apart. Each calf was bled at the time of each vaccination and 12 days following the second vaccination. The anti-leukotoxin titers were determined by a standard ELISA and are shown in Table 4.

Table 4
Anti-Leukotoxin Titers of Calves
Vaccinated with LKT 352

Group		Anti-Leukotoxin Titer at First Vaccination	Second Vaccination	10 Days After Second Vaccination
Controls	057	250	970	600
	065	3,500	10,000	20,000
	073	1,000	1,200	1,000
	081	230	200	230
	089	600	430	980
	097	500	500	500
Mean		1,013	2,216	3,885
LKT	352	2,500	150,000	100,000
	070	600	4,000	14,000
	078	1,900	18,000	25,000
	086	250	15,000	120,000
	094	700	1,100	130,000
	102	170	800	35,000
Mean		1,020	33,133	70,667

As can be seen, anti-leukotoxin titers were significantly higher in the LKT 352-treated group than the control calves at the time of the second vaccination and 10 days following the second vaccination.

The protective capacity of recombinantly produced LKT 352 combined with a saline extract of P. haemolytica was tested in a fourth vaccination trial (Vaccination Trial D). LKT 352 and P. haemolytica saline

extract (SE) were prepared using the general methods outlined above. The saline extract was found to have a protein concentration of 250 ug/ml. It was diluted with sterile double distilled water to a final volume of 1330 ml in order to adjust the protein concentration to 150 ug/ml. The recombinant LKT 352 contained 250 ug/ml of protein. Polyacrylamide gel electrophoresis revealed the presence of one major band and therefore, this antigen was used with no further dilution. Each dose of vaccine contained 100 ug of the new leukotoxin and 50 ug of saline extract.

Calves were vaccinated twice intramuscularly, 21 days apart with one of the following:

(1) Placebo; or

(2) _P. haemolytica_ subunit vaccine (LKT 352 plus SE) in Emulsigen Plus; or

(3) _P. haemolytica_ subunit vaccine in Avridine.

The experimental schedule was as follows:

Day -31	1st vaccination
Day -10	2nd vaccination
Day 0	Challenge with BHV-1
Day 4	Challenge with _P. haemolytica_
Day 5	Clinical observation ends

The results of this study can be seen in Table 5. As can be seen, twenty-five percent of the control calves died. In contrast, there was no mortality in the two groups given the subunit vaccine. The morbidity was also significantly lower in the subunit vaccine groups than in the placebo group (Fisher Exact Test $p < .05$).

Table 5

Results of Vaccination Trial D

Vaccine Group	n =	% Morbidity[a]	% Mortality[b]	Mean[c] Clinical Score	Mean[c] Temp (°C)	Mean[d] Sick Days	Mean Wt[e] Change (kg)
1. Placebo	8	100%	25%	1.04	40.3	4.5	-3.6
2. LKT 352 + SE in Emulsigen Plus	8	50%*	0%	0.36	39.4	1	+2.25
3. LKT 352 + SE in Avridine	8	50%*	0%	0.44	39.5	1	+3.75

[a] % of calves that developed a fever > 40.0 with clinical signs of BRD post P. haemolytica infection.

[b] % of calves that died of fibrinous pneumonia post P. haemolytica infection.

[c] Mean scores and temperatures of animals while alive.

[d] Mean days/calf that fever => 40.0 with clinical signs of BRD, calves that die are considered sick until end of trial.

[e] Mean change in weight (kg) from P. haemolytica infection until calf dies or trial ends.

* Morbidity was significantly lower ($P < 0.05$) than in the control group. Fisher's Exact Test.

A field trial (Vaccination Trial E) was carried out using the subunit vaccine comprised of LKT 352 and a P. haemolytica saline extract (SE). The vaccine formulations were as described in Vaccination Trial D.

The calves used were beef-type calves weighing from 250 kg to 325 kg. The calves were born during the spring, fall weaned, and purchased for the feedlot at auction markets. They were transported to the feedlot by truck and arrived within a few days of purchase.

Calves were randomly assigned to one of two vaccine groups. Calves in Group I were given a single 2 ml injection of the subunit Pasteurella vaccine intramuscularly. Calves in Group II were given a single 2 ml injection of placebo. The calves were processed at the time of arrival at the feedlot, and were assigned to one of the two treatment groups in rotation as they passed through a cattle chute. A technician administered the vaccines and recorded the treatment group to which each calf was assigned. A total of 2,324 calves were vaccinated, 1,168 in Group I and 1,156 in Group II.

Calves were kept and managed as typical feedlot animals. Feedlot cowboys were responsible for selecting and treating sick calves according to a protocol established by their consulting feedlot veterinarian. Selection of calves for treatment and post-mortem diagnosis was done without knowledge of the vaccination status of the calves. Records were maintained describing the daily diagnosis, temperature, and treatment of each sick calf. Calf health was monitored for 60 days after arrival. A gross post-mortem was done on all fatalities by a veterinarian within approximately 24 hrs of death and samples were submitted for further lab work if necessary. This information was used to establish morbidity (treatment) risks, and mortality risks. BRD morbidity risk scores were determined using the following equation:

$$\text{BRD Morbidity Risk} = \frac{\text{\# of calves sick with BRD in Group}}{\text{\# of calves in Group initially}}$$

The statistical significance of the differences between groups was established using risk ratios (or relative risk, RR), and by determining the 95% confidence intervals using the Taylor series confidence intervals when the comparison was between 2 groups. Risk ratios were established using the following equation:

$$\text{Risk Ratio (Relative Risk, RR)} = \frac{\text{Risk for One Group}}{\text{Risk for the Comparison Group}}$$

The significance of the differences was determined using the Mantel-Haenszel technique for summary risk ratios (MHRR) and the Greenland and Robins technique for calculating the 95% confidence intervals. All RRs were considered statistically significant if 95% confidence intervals did not include unity. When RRs and confidence intervals could not be calculated, the Fisher Exact 2-tailed test was used to determine the statistical significance between risks.

The results of this trial can be seen in Table 6. As is apparent, vaccination with LKT 352 in combination with a P. haemolytica saline extract (Group I) significantly reduced bovine respiratory disease morbidity and bovine respiratory disease mortality (all pneumonias) as compared to treatment with the placebo (Group II). The reduction in fibrinous pneumonia mortality was not significant at the 5% level. However, this is probably because a bovine herpesvirus-1 vaccine was also tested in combination with the Pasteurella vaccine. The BHV-1 vaccine appeared to cause immunosuppression which int rfered with response to the Pasteurella vaccine.

Table 6

Protection From Natural Bovine Respiratory Disease (Vaccination Trial E)

Group	BRD Morbidity	BRD Mortality	Fibrinous Pneumonia Mortality
I (Vaccine)	259/1168 22.2%[a]	6/1168 0.5%[a]	5/1168 0.4%
II (Placebo)	301/1156 26.0%	16/1156 1.4%	12/1156 1.0%

[a]Significantly lower ($P < 0.05$) than Group II

Identification of Neutralizing Epitopes of Leukotoxin

The *P. haemolytica* leukotoxin protein contains a series of repeated amino acid domains near the carboxy terminus. These domains are likely to be epitopes useful in vaccine compositions. The consensus amino acid sequence is Gly-Gly-X-Gly-X-Asp, where X is Lys, Asp, Val or Asn. (Highlander et al. (1989) DNA 8:15-28.) However, other substitutions likely to render immunologically active peptides include substitutions with an aliphatic amino acid, such as Gly, Ala, Val, Leu, Ile, a charged amino acid such as Asp, Glu, Arg, His or Lys, or a corresponding neutral amino acid such as Asn or Gln.

Based on this information, a synthetic peptide of the sequence GGNGDDFIDGGKGNDLLHGG was constructed by standard solid phase technology on an Applied Biosystems peptide synthesizer. Mice were immumnized with authentic leukotoxins prepared from either *P. haemolytica*, or *Actinobacillus pleuropneumoniae* (serotypes 1 and 5) at 100 micrograms per dose with Freund's Complete Adjuvant (first vaccination) or Freund's Incomplete Adjuvant (all subsequent vaccinations). High titer serum samples from immuniz d mice were tested, in a standard ELISA, for the following: (1) their ability to react with recombinant

and authentic P. haemolytica leukotoxin; (2) their ability to react with the toxin produced by A. pleuropneumoniae; and (3) their ability to react with the synthetic peptide described above. The results, summarized in Table 7, are expressed as the relative reactivity at a serum dilution of 1 in 100,000.

Table 7

Presence of Synthetic Peptide Epitopes in Toxins from P. haemolytica and A. pleuropneumonia serotypes 1 and 5

	Relative Serological Response To:		
Toxin Prepared From:	Synthetic Peptide	Actinobacillus Toxin	Pasteurella Toxin
A. pleuropneumoniae sero.5	+++	++++	++
A. pleuropneumoniae sero.1	+	++++	+
P. haemolytica	+++	not determined	++++

This data indicated that animals vaccinated with either of the three leukotoxins developed antibodies which reacted with all toxins and a synthetic peptide based on a portion of the P. haemolytica toxin. Once an appropriate level of anti-peptide serum antibody was reached (ELISA titer of 100,000 or greater), spleen cells were fused with NS1 cells and monoclonal antibody-producing clones were isolated by standard techniques. Culture supernatants from these clones were tested for their ability to react with the synthetic peptide (above) and the respective toxins in an ELISA assay. The results for 2 clones are shown in Table 8.

Table 8

Clone	Immunogen	Relative Reaction With: Pasteurella Toxin	Synthetic Peptide	Actino-bacillus Toxin
ET122-6A4-3	Pasteurella toxin	++++	+++++	ND[1]
N37-3F9-6	Actinobacillus toxin	ND	++++	+++++

[1]Not determined

These results demonstrate that each of these monoclonal antibodies react with an epitope which is shared by the P. haemolytica and A. plauropneumoniae toxins, and that this epitope is structurally similar to that of the synthetic peptide. This peptide is also structurally similar to a bovine rotavirus synthetic peptide of the sequence T M N G N E F Q T G G I G N L P I R N W N A C, representing amino acids 40-60 of the VP6 protein. the monoclonal antibodies described above can therefore be used to determine the degree of their cross-reactivity with rotavirus proteins based on the epitope represented by the synthetic peptides. furthermore, the immunologically active leukotoxin fragments might prove useful in immunizing agains rotavirus.

The antibodies above can also be tested for (1) their ability to react with and neutralize other similar toxins, including those produced by E. coli, Proteus vulgaris, Proteus mirabilis and Actinobacillus actinomycetemcomitans. A DNA sequence coding for this epitope can be cloned and expressed in either E. coli, S. aureus or Baculovirus.

Example 2

P. haemolytica fimbriae were id ntified, purified and the protective capacity determined as described below.

1. Identification and Purification of Fimbriae

A crude shear fraction of P. haemolytica fimbriae was obtained in the following manner. P. haemolytica A1 strain B122 was grown on brain heart infusion agar for 13 hours at 37°C in Nunc bioassay dishes (Gibco). The cells were then harvested using 20 ml phosphate buffered saline (PBS), pH 7.4, per plate and sheared by blending for 5 minutes in an Osterizer blender. Sheared cells were pelleted by centrifugation and the supernatant was examined by electron microscopy by placing samples on formvar-coated, carbon-stabilized copper grids. The samples were negatively stained with 1% uranyl acetate and examined with a Philips EM-410LS electron microscope operating at 60 kV. The structures obtained are shown in Figure 6A and were referred to as "PH-K fimbriae." These structures were approximately 12 nm in diameter and varied in length to greater than 1000 nm.

SDS-polyacrylamide gel electrophoresis of this crude shear fraction was performed in a 12.5% (w/v) polyacrylamide gel (1.0 mm) with a Protean II vertical gel unit (Bio-Rad Laboratories, Richmond, CA). The SDS buffer system used was that described by the manufacturer. Several bands were present. A 35 kD protein was particularly abundant.

The crude shear fraction isolated above was further purified by centrifugation through a CsCl step gradient. Specifically, the crude shear fraction was centrifuged at 25,000 rpm overnight in a Beckman SW27 rotor. The pellet was dissolved in a small quantity of 10 mM Tris-HCl, pH 7.5 and applied to the top of a 1.0-1.5 g/ml CsCl gradient and centrifuged for 24-36 hours at 12°C

at 45,000 rpm in a Beckman 50Ti rotor. Two major bands were visible. Electron microscopy (as described above) of the lower band revealed largely membrane blebs while the upper band (density = 1.32 g/ml) was composed of PH-K fimbriae as depicted in Figure 6B.

The fimbrial fraction was withdrawn and dialyzed overnight against 10 mM Tris-HCl, pH 7.5 and the centrifugation repeated. In some experiments, the fimbrial fraction was incubated in 5M urea at 37^{o}C for 5 hours prior to the second CsCl ultracentrifugation. Only one band at a density of 1.32 g/ml was observed. This fraction was further examined by polyacrylamide gel electrophoresis and electron microscopy as described above. This fraction appeared to contain pure PH-K fimbriae as depicted in Figure 6C. The fimbriae were approximately 12 nm in diameter and varied in length to more than 500 nm. Only one band was present on SDS-PAGE gels, corresponding to a molecular weight of 35,000.

Monoclonal antibodies were raised against native fimbriae via the following method to test whether CsCl-purified fraction was indeed the PH-K subunit. BALB/c mice were immunized with 5 ug of purified fimbriae, boosted twice at approximately 2 weeks apart, and their spleens removed. Spleen cells were fused with NS-1 cells using the procedure described by Sabara et al. (1987) J. Gen. Virol. 68:123-133. Samples were screened by immunoblotting the purified fimbriae on SDS-PAGE gels as described by Sabara et al., supra. The monoclonal antibodies reacted only with the CsCl purified fraction on immunoblots, indicating that this represented the PH-K subunit.

To determine the isoelectric point of the purified fimbriae, the filaments were subjected to chromatography on a chromatofocusing column by the following method. A PBE 94 column (3 X 60 cm, Pharmacia Fine Chemicals, Uppsala, Sweden), was equilibrated with 25 mM

histidine-HCl, pH 6.2. One hundred to five hundred ug of purified fimbriae were applied to the equilibrated column. The fimbriae were eluted with Polybuffer 74-HCl, pH 4.0 (Pharmacia) as described by the manufacturer. As illustrated in Figure 7, one peak was eluted at pH 4.8. This pI value was confirmed by isoelectric focusing using a Phast Gel system (Pharmacia).

The hemagglutination properties of P. haemolytica whole cells, the crude shear fraction and the CsCl-purified fimbrial fraction were tested in the following manner. P. haemolytica A1 strain B122 was grown on brain heart infusion agar as described above. Cells were grown to a density of 10^{10} per ml and harvested in PBS. Serial dilutions were prepared. Bovine erythrocytes were washed three times with PBS and suspended at a concentration of 5% (v/v). 75 ul of the washed erythrocytes were added to equal volumes of diluted bacteria in microtiter plate wells (Costar, 96 well, Cambridge, MA). The plates were incubated for 24 hours at $37^{o}C$ and 14 hours at $4^{o}C$. The hemagglutination titer was the reciprocal of the highest dilution which exhibited a positive response.

Crude and purified fimbrial fractions were used in a hemagglutination assay as described above at a concentration of 1 mg/ml. Neutralization assays were conducted by preincubating the crude or purified fimbriae for 1 hour at room temperature with serum (1/50) from a calf which had survived a natural P. haemolytica infection. As illustrated in Table 9, antisera to P. haemolytica surface components was able to neutralize hemagglutination by whole cells and the crude shear fraction. However, CsCl-purified fimbriae exhibited no activity.

Table 9

Hemagglutination of Bovine Erythrocytes by P. Haemolytica Strain b122

Fraction	HA Titer
Cells	64
Cells plus antisera[a]	2
Crude shear fraction	32
Crude shear fraction plus antisera	0
Purified fimbriae	0

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[a]Antisera was from a calf which had recovered from a natural P. haemolytica infection.

The CsCl-purified fimbrial protein was sequenced using standard amino terminal sequencing techniques. The sequence was found to be 'xxxxxx-Ile-Ala-Ala-Leu-Asn-Thr-Leu-Asn-Arg-Leu-Ser-Ala-Asn-Asn-Gly-Ala-Ser-Gln-Lys-Asn-(Met or Phe).

Production of P. haemolytica Recombinant Fimbrial Protein

A nucleic acid probe based on the amino-terminal protein sequence disclosed above can be prepared. The sequence of the synthetic probe is

5'-CAA/GAAA/GAATATGGAA/GAAA/GTT-3'.

The probe can be labeled with ^{32}P and hybridized to Southern blotted P. haemolytica genomic DNA digested with various restriction endonucleases. The fragment can be cloned into the vector lambda-ZAP and identified by colony hybridization with the above oligonucleotide probe. The cloned DNA containing the area of hybridization can be

sequenced in order to confirm that it codes for the same amino acid sequence as determined above. Subclones can be constructed and the entire gene sequenced. The fimbrial protein gene can be expressed in any suitable expression system known in the art.

2. Vaccine Compositions Made from Purified Fimbriae

In a first trial, vaccines were made by mixing 100 ug in 1.0 ml of 0.1 M PBS, pH 7.2, of the CsCl-purified fimbriae with 1.0 ml of avridine. Six calves were injected intramuscularly (I.M.) with the vaccine composition, six calves were injected intratracheally (I.T.), and a third group of six calves was injected I.M. with a control substance which contained avridine but no fimbrial protein. The animals were boosted 24 days later. Ten days after boosting, the animals were challenged with Bovine herpes virus-1, followed four days later by exposure to P. haemolytica A1 strain B122. The calves were checked daily for clinical signs of pneumonia, temperature and feeding. In addition, weight loss and lung scores were recorded at necropsy. The results are summarized in Tables 10-12 below and Figures 8 and 9.

Table 10

Mean ELISA Titers (Vaccine Trial #1) of Calves Immunized with P. haemolytica PH-K Fimbria

GROUP	MEAN TITER			
	DAY 0	DAY 14	DAY 21	DAY 32
VACCINATED I.M.	2,650	18,000	15,600	58,200
VACCINATED I.T.	1,433	3,667	4,783	10,500
CONTROL	425	1,208	5,267	8,267

I.M.= vaccinated intramuscularly
I.T.= vaccinated intratracheally
Control = Avridine

Table 11

P. haemolytica Pili Vaccine Experiment: Clinical Scores

(Vaccine Trial #1)

Calf ID	Treatment	Cumulative Clinical Score	Total Sick Days	Outcome	Lung Score
276	Phaem Fimbriae (100 ug IM)	32	0	Survived	0
277	Phaem Fimbriae (100 ug IM)	45	0	Survived	0
278	Phaem Fimbriae (100 ug IM)	43	0	Survived	0
279	Phaem Fimbriae (100 ug IM)	102	4	Died	26
280	Phaem Fimbriae (100 ug IM)	45	0	Survived	0
	Mean	53.4	4	4/5 Survived	mean=5.2 median=0
282	Phaem Fimbriae (100 ug IT)	42	0	Survived	0
283	Phaem Fimbriae (100 ug IT)	61	2	Survived	0
284	Phaem Fimbriae (100 ug IT)	97	4	Died	19
285	Phaem Fimbriae (100 ug IT)	122	4	Died	18
286	Phaem Fimbriae (100 ug IT)	57	0	Survived	0
287	Phaem Fimbriae (100 ug IT)	55	0	Survived	0
	Mean	78.4	10	4/5 Survived	mean=6.2 median=0
288	Control (Avridine)	40	0	Survived	0
289	Control (Avridine)	56	1	Survived	0
290	Control (Avridine)	113	5	Died	11
291	Control (Avridine)	63	1	Survived	0
292	Control (Avridine)	49	0	Survived	0
293	Control (Avridine)	133	6	Died	14
	Mean	82.8	13	4/6 Survived	mean=4.2 median=0

Table 12

P. haemolytica Vaccine Experiment: Weight Changes

(Vaccine Trial #1)

Calf ID	Treatment		Initial Weight (kg)	Final Weight (kg)	Weight Change
276	Phaem fimbriae	Intramuscular	170	176	6
277	Phaem fimbriae	Intramuscular	168	158	-10
278	Phaem fimbriae	Intramuscular	156	147	-9
279	Phaem fimbriae	Intramuscular	185	172	-13
280	Phaem fimbriae	Intramuscular	190	185	-5
281	Phaem fimbriae	Intramuscular	Excluded from study		
					Mean -6.2
282	Phaem fimbriae	Intratracheal	166	169	3
283	Phaem fimbriae	Intratracheal	174	162	-12
284	Phaem fimbriae	Intratracheal	208	186	-22
285	Phaem fimbriae	Intratracheal	195	192	-3
286	Phaem fimbriae	Intratracheal	183	169	-14
287	Phaem fimbriae	Intratracheal	172	167	-5
					Mean -8.8
288	Avridine Control		230	228	-2
289	Avridine Control		168	158	-10
290	Avridine Control		155	156	1
291	Avridine Control		225	209	-16
292	Avridine Control		196	195	-1
293	Avridine Control		183	170	-13
					Mean -6.8

As can be seen, serum antibody titers were lowest in the control group and highest in the group which received the fimbriae vaccine I.M. Calves vaccinated I.T. did not have significantly higher titers than the control group. The clinical signs of pneumonia and number of sick days was lower in the I.M. fimbriae vaccinated group than in the control group.

A second vaccine trial was carried out. One group of 7 calves was administered the vaccine composition I.M. as described above containing the purified fimbrial protein. A second group of six calves received a control composition containing avridine only. The calves were boosted 22 days later and challenged with bovine herpes virus-1 15 days after being boosted. The calves were then exposed to _P. haemolytica_ 4 days later. The results are summarized in Figures 10-13. The fimbrial-vaccinated group was clearly protected against experimental challenge with _P. haemolytica_ as reflected in lower mortality, clinical scores and lung scores. Only one calf in the vaccinated group had a significant lung score.

Thus _P. haemolytica_ purified fimbrial protein shows utility in a vaccine against shipping fever. Vaccination may reduce colonization of the lung resulting in decreased morbidity and mortality. It is likely that a subunit vaccine derived from the recombinant fimbrial protein will also be useful for this purpose.

Example 3

Plasmin receptors on the outer membrane of _P. haemolytica_ A1 strain B122 were identified by running fibrin plate assays since plasmin has the ability to lyse fibrin. Fibrin plates were prepared by clotting 10 ml of 0.1% bovine fibrinogen (Sigma) in phosphate-buffered saline, pH 7.2, with 0.2 ml of bovine thrombin (10 NIH U/ml) in 0.5 M $CaCl_2$. Bacteria were prepared by growing _P. haemolytica_ A1 strain B122 overnight at 37°C in brain

heart infusion agar, performing a 100-fold dilution and growing the diluted culture until it reached log-phase (approximately 6 hours). The bacteria were then washed once and 0.2 g (weight/volume) of the washed cells were incubated with 50 picomoles of bovine plasmin in 2 ml of VBS-gel (Veronal-buffered saline, pH 7.35 containing 1.0 mM $MgCl_2$, 0.15 mM $CaCl_2$ and 0.1% gelatin). The reaction was allowed to proceed for 45 minutes at 37°C after which the mixture was centrifuged at 2500 rpm for 15 minutes with two washes in VBS-gel. The washed cell pellet was resuspended in 400 microliters VBS-gel and 50 microtiter samples were spotted on the fibrin plates prepared above. A control of free plasmin was included. The reaction proceeded overnight in a moist chamber at 37°C. The fibrin clot was clearly hydrolyzed by log-phase bacteria. The degree of hydrolysis of the fibrin clot was scored by measuring the area of the zone of clearing from the under-side of the plate.

The above experiment was repeated with cells in log-phase as well as with cells in stationary-phase. No clot hydrolysis was observed with the stationary-phase cells indicating that the plasmin receptor is expressed only by actively growing cells.

Plasmin is able to degrade a large number of substrates, including casein. Therefore, the above experiment was repeated using skim milk-agarose plates and log-phase bacterial cultures. Lysis was visible after incubation at 37°C, however not to the same degree as observed with the fibrin medium.

To test the ability of P. haemolytica serotypes 1-11 to bind plasmin, these serotypes were tested using skim milk-agarose plates as described above. All serotypes were able to degrade this substrate, although the results varied widely. The results can be seen in Table 13.

Table 13

Plasmin-Binding Capacity of Different P. haemolytica Serotypes

Strain[1]	Serotype	Plasmin-binding[2]
PH45	1	+++
PH46	1	++
PH47	2	++/-
PH48	3	+/-
PH49	4	++
PH50	6	+++
PH51	7	++
PH52	8	++++
PH53	9	++/-
PH54	5	++++/-
PH55	10	+
PH56	11	++++

[1] Strain PH45 is the same as B122 and is from the VIDO culture collection. All others were obtained from the American Type Culture Collection.

[2] Plasmin-binding was scored by the relative amounts of lysis of skim milk.

The receptor protein was identified by separating outer membrane proteins from a serotype 1 isolate of P. haemolytica on an SDS-polyacrylamide gel. The components were electroblotted to a nitrocellulose membrane and reacted with biotinylated plasmin 50-200 ng/ml (Sigma). Strepavidin-alkaline phosphatase was added to the membrane and the membrane stained using nitroblue tetrazolium and bromo-chloro-indolyl phosphate. One band corresponding to an approximate molecular weight of 41,000

was visible. This corresponds to a minor outer membrane component (see Figure 14).

Plasminogen is the inactive zymogen precursor of plasmin. To test whether P. haemolytica was able to bind plasminogen, the above experiment was repeated using biotinylated plasminogen. Identical results were obtained indicating that this bacterium has the ability to bind both plasmin and plasminogen.

Production of P. haemolytica Recombinant Plasmin Receptor

A P. haemolytica A1 gene library was constructed in E. coli using the cosmid vector pHC79. The library was screened for clones able to bind biotinylated bovine plasmin. The resulting positive clones were tested for their ability to bind plasmin and subsequently degrade a fibrin clot. Positive recombinants can be subcloned and the coding region sequenced. The plasmin receptor gene can be expressed in E. coli, baculovirus and other expression systems known in the art.

The plasmin/plasminogen receptor of P. haemolytica may function as a virulence determinant by permitting rapid penetration of the organism in the lower respiratory tract. Thus, immunization with this protein may result in protective immunity by blocking subsequent colonization of the lung. Additionally, monospecific antisera or the cloned gene may be useful in diagnostic tests for P. haemolytica.

Thus, P. haemolytica purified and recombinant proteins for use in stimulating immunity against pneumonia and other respiratory diseases have been disclosed. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the

spirit and the scope of the invention as defined by the appended claims.

We claim:

1. A vaccine composition comprising a pharmaceutically acceptable vehicle and a subunit antigen composition, said subunit antigen composition comprising at least one immunogenic polypeptide including an immunogenic amino acid sequence of a P. haemolytica protein, or an amino acid sequence substantially homologous and functionally equivalent thereto, selected from the group consisting of:

(a) P. haemolytica fimbrial protein;

(b) P. haemolytica plasmin receptor protein;

(c) P. haemolytica 50K outer membrane protein; and

(d) P. haemolytica leukotoxin.

2. The vaccine composition of claim 1 wherein the composition further comprises a saline extract of P. haemolytica.

3. The vaccine composition of claims 1 or 2 wherein said P. haemolytica leukotoxin is leukotoxin 352 (LKT 352).

4. The vaccine composition of claims 1, 2 or 3 further comprising an adjuvant.

5. The vaccine composition of claim 1 wherein said immunogenic amino acid sequence of leukotoxin comprises Gly-Gly-X-Gly-X-Asp, wherein X is selected from the group consisting of an aliphatic amino acid, and a charged amino acid or its corresponding neutral amino acid.

6. The vaccine composition of claim 5 wherein X is selected from the group consisting of Lys, Asp, Val, and Asn.

7. The vaccine composition of claim 5 wherein said immunogenic amino acid sequence comprises GGNGDDFIDGGKGNDLLHGG.

8. Isolated P. haemolytica fimbrial protein.

9. Isolated P. haemolytica plasmin receptor protein.

10. Isolated P. haemolytica 50K outer membrane protein.

11. Isolated P. haemolytica leukotoxin 352 (LKT 352).

12. A DNA construct comprising an expression cassette comprised of:

(a) a DNA coding sequence for a polypeptide containing at least one epitope of a P. haemolytica protein selected from the group consisting of P. haemolytica fimbrial protein, P. haemolytica plasmin receptor protein, and P. haemolytica 50K outer membrane protein; and

(b) control sequences that are operably linked to said coding sequence whereby said coding sequence can be transcribed and translated in a host cell, and at least one of said control sequences is heterologous to said coding sequence.

13. Plasmid pAA352.

14. A host cell stably transformed by a DNA construct according to claims 12 or 13.

15. A method of producing a recombinant polypeptide comprising:

(a) providing a population of host cells according to claim 14; and

(b) growing said population of cells under conditions whereby the polypeptide encoded by said expression cassette is expressed.

16. A composition for use in preventing or ameliorating respiratory disease in a ruminant which comprises as an active ingredient at least one immunogenic polypeptide including an immunogenic amino acid sequence of a _P. haemolytica_ protein, or an amino acid sequence substantially homologous and functionally equivalent thereto, selected from the group consisting of:

(a) _P. haemolytica_ fimbrial protein;

(b) _P. haemolytica_ plasmin receptor protein;

(c) _P. haemolytica_ 50K outer membrane protein; and

(d) _P. haemolytica_ leukotoxin,

in admixture with a pharmaceutically acceptable vehicle.

17. A method of making polyclonal antiserum to _P. haemolytica_ comprising administering a composition according to claim 1 to a mammal, and recovering polyclonal serum from said mammal.

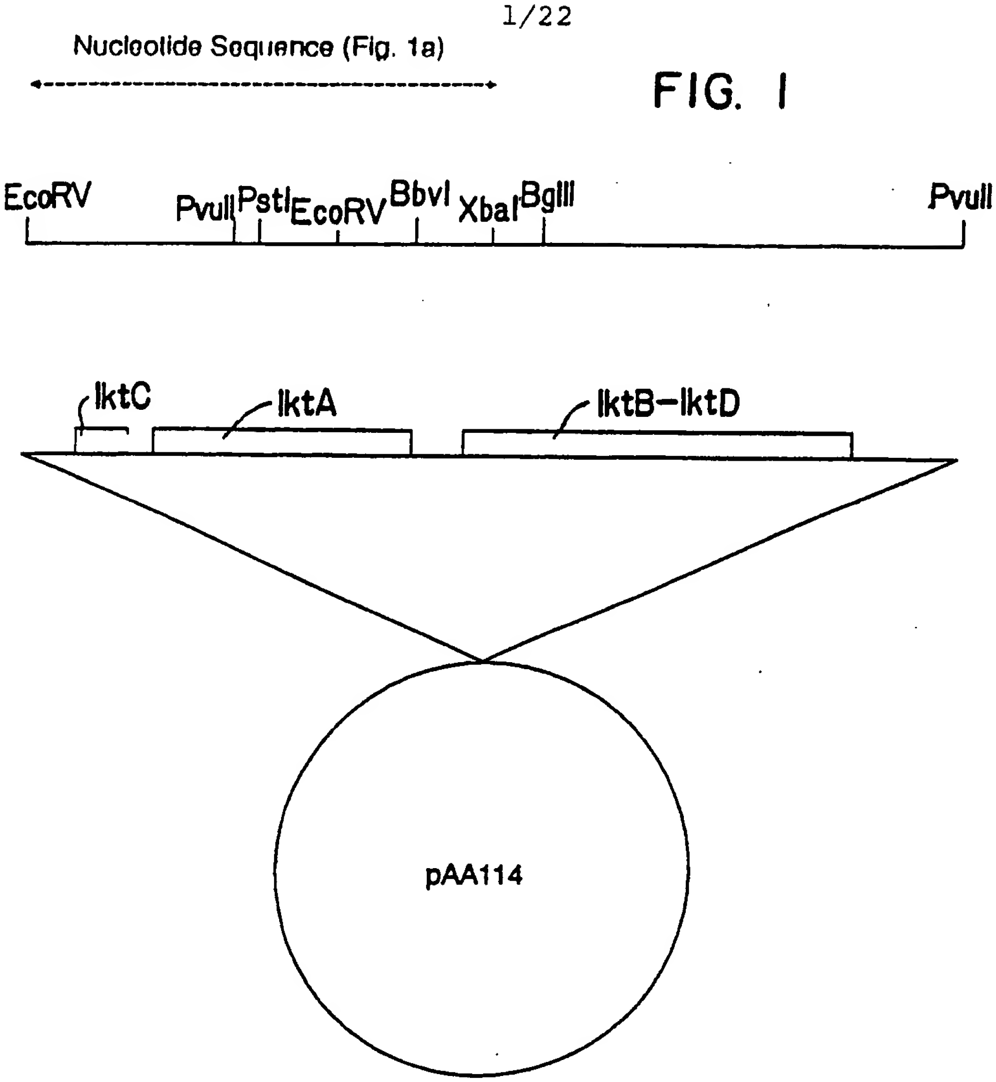
Nucleotide Sequence (Fig. 1a)
EcoRV
PvuII
PstI
EcoRV
BbvI
XbaI
BglII
PvuII
lktC
lktA
lktB-lktD
pAA114

FIG. 1

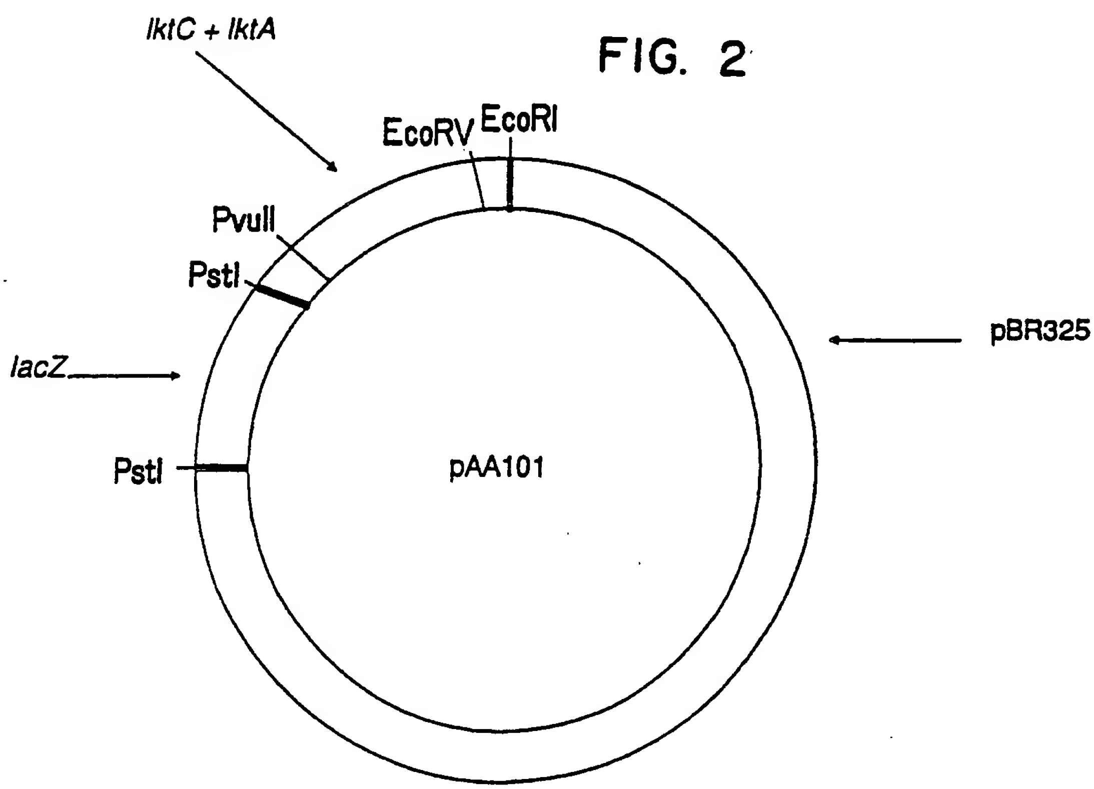
lktC + lktA
EcoRV
EcoRI
PvuII
PstI
lacZ
PstI
pAA101
pBR325

FIG. 2

FIG.3-1

```
  1 MGTRLTTLSNGLKNTLTATKSGLHKAGQSLTQAGSSLKTGAKKIILYIPQNYQYDTEQGNGLQDLVKAA
 70 EELGIEVQREERNNIATAQTSLGTIQTAIGLTERGIVLSAPQIDKLLQKTKAGQALGSAESIVQNANKA
139 KTVLSGIQSILGSVLAGMDLDEALQNNSNQHALAKAGLELTNSLIENIANSVKTLDEFGEQISQFGSKL
208 QNIKGLGTLGDKLKNIGGLDKAGLGLDVISGLLSGATAALVLADKNASTAKKVGAGFELANQVVGNITK
277 AVSSYILAQRVAAGLSSTGPVAALIASTVSLAISPLAFAGIADKFNHAKSLESYAERFKKLGYDGDNLL
346 AEYQRGTGTIDASVTAINTALAAIAGGVSAAAGRRIRGIPGDPVVLQRRDWENPGVTQLNRLAAHPPFA
415 SWRNSEEARTDRPSQQLRSLNGEWRFAWFPAPEAVPESWLECDLPEADTVVVPSNWQMHGYDAPIYTNV
484 TYPITVNPPFVPTENPTGCYSLTFNVDESWLQEGQTRIIFDGVNSAFHLWCNGRWVGYGQDSRLPSEFD
553 LSAFLRAGENRLAVMVLRWSDGSYLEDQDMWRMSGIFRDVSLLHKPTTQISDFHVATRFNDDFSRAVLE
622 AEVQMCGELRDYLRVTVSLWQGETQVASGTAPFGGEIIDERGGYADRVTLRLNVENPKLWSAEIPNLYR
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691 AVVELHTADGTLIEAEACDVGFREVRIENGLLLLNGKPLLIRGVNRHEHHPLHGQVMDEQTMVQDILLM

760 KQNNFNAVRCSHYPNHPLWYTLCDRYGLYVVDEANIETHGMVPMNRLTDDPRWLPAMSERVTRMVQRDR

829 NHPSVIIWSLGNESGHGANHDALYRWIKSVDPSRPVQYEGGGADTTATDIICPMYARVDEDQPFPAVPK

898 WSIKKWLSLPGETRPLILCEYAHAMGNSLGGFAKYWQAFRQYPRLQGGFVWDWVDQSLIKYDENGNPWS

967 AYGGDFGDTPNDRQFCMNGLVFADRTPHPALTEAKHQQQFFQFRLSGQTIEVTSEYLFRHSDNELLHWM

1036 VALDGKPLASGEVPLDVAPQGKQLIELPELPQPESAGQLWLTVRVVQPNATAWSEAGHISAWQQWRLAE

1105 NLSVTLPAASHAIPHLTTSEMDFCIELGNKRWQFNRQSGFLSQMWIGDKKQLLTPLRDQFTRAPLDNDI

1243 QMAITVDVEVASDTPHPARIGLNCQLAQVAERVNWLGLGPQENYPDRLTAACFDRWDLPLSDMYTPYVF

1312 PSENGLRCGTRELNYGPHQWRGDFQFNISRYSQQQLMETSHRHLLHAEEGTWLNIDGFHMGIGGDDSWS

1381 PSVSAEFQLSAGRYHYQLVWCQK

FIG.3-2

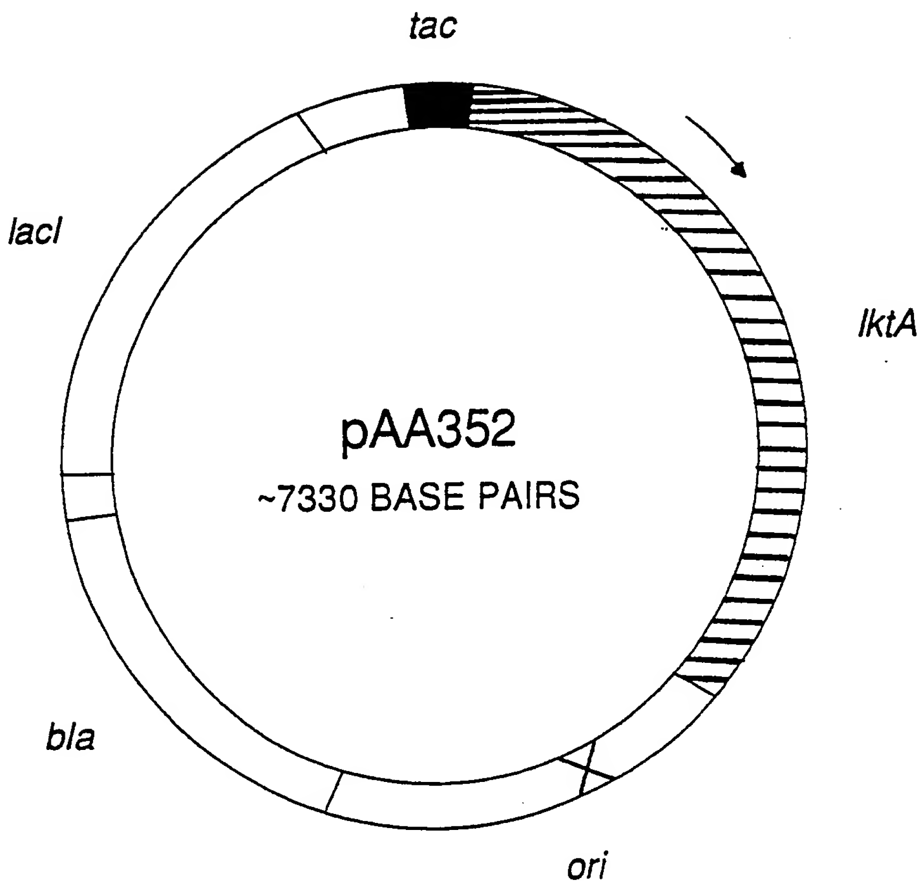

FIG. 4

FIG.5-1

```
        10          20          30          40          50          60          70          80          90
    *    *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
ATG GCT ACT GTT ATA GAT CTA AGC TTC CCA AAA ACT GGG GCA AAA AAA ATT ATC CTC TAT ATT CCC CAA AAT TAC CAA TAT GAT ACT GAA
TAC CGA TGA CAA TAT CTA GAT TCG AAG GGT TTT TGA CCC CGT TTT TTT TAA TAG GAG ATA TAA GGG GTT TTA ATG GTT ATA CTA TGA CTT
Met Ala Thr Val Ile Asp Leu Ser Phe Pro Lys Thr Gly Ala Lys Lys Ile Ile Leu Tyr Ile Pro Gln Asn Tyr Gln Tyr Asp Thr Glu>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_t___c___c___c___c___c___c___c___c___c___c___>
___a___a___VECTOR SEQUENCE_a___a___a__>

       100         110         120         130         140         150         160         170         180
    *    *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
CAA GGT AAT GGT TTA CAG GAT TTA GTC AAA GCG GCC GAA GAG TTG GGG ATT GAG GTA CAA AGA GAA GAA CGC AAT AAT ATT GCA ACA GCT
GTT CCA TTA CCA AAT GTC CTA AAT CAG TTT CGC CGG CTT CTC AAC CCC TAA CTC CAT GTT TCT CTT CTT GCG TTA TTA TAA CGT TGT CGA
Gln Gly Asn Gly Leu Gln Asp Leu Val Lys Ala Ala Glu Glu Leu Gly Ile Glu Val Gln Arg Glu Glu Arg Asn Asn Ile Ala Thr Ala>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

       190         200         210         220         230         240         250         260         270
    *    *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
CAA ACC AGT TTA GGC ACG ATT CAA ACC GCT ATT GGC TTA ACT GAG CGT GGC ATT GTG TTA TCC GCT CCA CAA ATT GAT AAA TTG CTA CAG
GTT TGG TCA AAT CCG TGC TAA GTT TGG CGA TAA CCG AAT TGA CTC GCA CCG TAA CAC AAT AGG CGA GGT GTT TAA CTA TTT AAC GAT GTC
Gln Thr Ser Leu Gly Thr Ile Gln Thr Ala Ile Gly Leu Thr Glu Arg Gly Ile Val Leu Ser Ala Pro Gln Ile Asp Lys Leu Leu Gln>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>
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FIG.5-2

```
  280                 290                 300                 310                 320                 330                 340                 350                 360
    *         *         *         *         *         *         *         *         *         *         *         *         *         *         *         *         *         *
AAA ACT AAA GCA GGC CAA GCA TTA GGT TCT GCC GAA AGC ATT GTA CAA AAT GCA AAT AAA GCC AAA ACT GTA TTA TCT GGC ATT CAA TCT
TTT TGA TTT CGT CCG GTT CGT AAT CCA AGA CGG CTT TCG TAA CAT GTT TTA CGT TTA TTT CGG TTT TGA CAT AAT AGA CCG TAA GTT AGA
Lys Thr Lys Ala Gly Gln Ala Leu Gly Ser Ala Glu Ser Ile Val Gln Asn Ala Asn Lys Ala Lys Thr Val Leu Ser Gly Ile Gln Ser>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

            370                 380                 390                 400                 410                 420                 430                 440                 450
    *         *         *         *         *         *         *         *         *         *         *         *         *         *         *         *         *         *
ATT TTA GGC TCA GTA TTG GCT GGA ATG GAT TTA GAT GAG GCC TTA CAG AAT AAC AGC AAC CAA CAT GCT CTT GCT AAA GCT GGC TTG GAG
TAA AAT CCG AGT CAT AAC CGA CCT TAC CTA AAT CTA CTC CGG AAT GTC TTA TTG TCG TTG GTT GTA CGA GAA CGA TTT CGA CCG AAC CTC
Ile Leu Gly Ser Val Leu Ala Gly Met Asp Leu Asp Glu Ala Leu Gln Asn Asn Ser Asn Gln His Ala Leu Ala Lys Ala Gly Leu Glu>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

            460                 470                 480                 490                 500                 510                 520                 530                 540
    *         *         *         *         *         *         *         *         *         *         *         *         *         *         *         *         *         *
CTA ACA AAT TCA TTA ATT GAA AAT ATT GCT AAT TCA GTA AAA ACA CTT GAC GAA TTT GGT GAG CAA ATT AGT CAA TTT GGT TCA AAA CTA
GAT TGT TTA AGT AAT TAA CTT TTA TAA CGA TTA AGT CAT TTT TGT GAA CTG CTT AAA CCA CTC GTT TAA TCA GTT AAA CCA AGT TTT GAT
Leu Thr Asn Ser Leu Ile Glu Asn Ile Ala Asn Ser Val Lys Thr Leu Asp Glu Phe Gly Glu Gln Ile Ser Gln Phe Gly Ser Lys Leu>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

            550                 560                 570                 580                 590                 600                 610                 620                 630
    *         *         *         *         *         *         *         *         *         *         *         :         *         *         *         *         *         *
CAA AAT ATC AAA GGC TTA GGG ACT TTA GGA GAC AAA CTC AAA AAT ATC GGT GGA CTT GAT AAA GCT GGC CTT GGT TTA GAT GTT ATC TCA
GTT TTA TAG TTT CCG AAT CCC TGA AAT CCT CTG TTT GAG TTT TTA TAG CCA CCT GAA CTA TTT CGA CCG GAA CCA AAT CTA CAA TAG AGT
Gln Asn Ile Lys Gly Leu Gly Thr Leu Gly Asp Lys Leu Lys Asn Ile Gly Gly Leu Asp Lys Ala Gly Leu Gly Leu Asp Val Ile Ser>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>
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FIG.5-3

```
        640         650         660         670         680         690         700         710         720
  *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GGG CTA TTA TCG GGC GCA ACA GCT GCA CTT GTA CTT GCA GAT AAA AAT GCT TCA ACA GCT AAA AAA GTG GGT GCG GGT TTT GAA TTG GCA
CCC GAT AAT AGC CCG CGT TGT CGA CGT GAA CAT GAA CGT CTA TTT TTA CGA AGT TGT CGA TTT TTT CAC CCA CGC CCA AAA CTT AAC CGT
Gly Leu Leu Ser Gly Ala Thr Ala Ala Leu Val Leu Ala Asp Lys Asn Ala Ser Thr Ala Lys Lys Val Gly Ala Gly Phe Glu Leu Ala>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

        730         740         750         760         770         780         790         800         810
  *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
AAC CAA GTT GTT GGT AAT ATT ACC AAA GCC GTT TCT TCT TAC ATT TTA GCC CAA CGT GTT GCA GCA GGT TTA TCT TCA ACT GGG CCT GTG
TTG GTT CAA CAA CCA TTA TAA TGG TTT CGG CAA AGA AGA ATG TAA AAT CGG GTT GCA CAA CGT CGT CCA AAT AGA AGT TGA CCC GGA CAC
Asn Gln Val Val Gly Asn Ile Thr Lys Ala Val Ser Ser Tyr Ile Leu Ala Gln Arg Val Ala Ala Gly Leu Ser Ser Thr Gly Pro Val>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

        820         830         840         850         860         870         880         890         900
  *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GCT GCT TTA ATT GCT TCT ACT GTT TCT CTT GCG ATT AGC CCA TTA GCA TTT GCC GGT ATT GCC GAT AAA TTT AAT CAT GCA AAA AGT TTA
CGA CGA AAT TAA CGA AGA TGA CAA AGA GAA CGC TAA TCG GGT AAT CGT AAA CGG CCA TAA CGG CTA TTT AAA TTA GTA CGT TTT TCA AAT
Ala Ala Leu Ile Ala Ser Thr Val Ser Leu Ala Ile Ser Pro Leu Ala Phe Ala Gly Ile Ala Asp Lys Phe Asn His Ala Lys Ser Leu>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>
```

FIG.5—4

```
         910       920       930       940       950       960       970       980       990
          *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GAG AGT TAT GCC GAA CGC TTT AAA AAA TTA GGC TAT GAC GGA GAT AAT TTA TTA GCA GAA TAT CAG CGG GGA ACA GGG ACT ATT GAT GCA
CTC TCA ATA CGG CTT GCG AAA TTT TTT AAT CCG ATA CTG CCT CTA TTA AAT AAT CGT CTT ATA GTC GCC CCT TGT CCC TGA TAA CTA CGT
Glu Ser Tyr Ala Glu Arg Phe Lys Lys Leu Gly Tyr Asp Gly Asp Asn Leu Leu Ala Glu Tyr Gln Arg Gly Thr Gly Thr Ile Asp Ala>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

        1000      1010      1020      1030      1040      1050      1060      1070      1080
          *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
TCG GTT ACT GCA ATT AAT ACC GCA TTG GCC GCT ATT GCT GGT GGT GTG TCT GCT GCT GCA GCC GGC TCG GTT ATT GCT TCA CCG ATT GCC
AGC CAA TGA CGT TAA TTA TGG CGT AAC CGG CGA TAA CGA CCA CCA CAC AGA CGA CGA CGT CGG CCG AGC CAA TAA CGA AGT GGC TAA CGG
Ser Val Thr Ala Ile Asn Thr Ala Leu Ala Ala Ile Ala Gly Gly Val Ser Ala Ala Ala Ala Gly Ser Val Ile Ala Ser Pro Ile Ala>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

        1090      1100      1110      1120      1130      1140      1150      1160      1170
          *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
TTA TTA GTA TCT GGG ATT ACC GGT GTA ATT TCT ACG ATT CTG CAA TAT TCT AAA CAA GCA ATG TTT GAG CAC GTT GCA AAT AAA ATT CAT
AAT AAT CAT AGA CCC TAA TGG CCA CAT TAA AGA TGC TAA GAC GTT ATA AGA TTT GTT CGT TAC AAA CTC GTG CAA CGT TTA TTT TAA GTA
Leu Leu Val Ser Gly Ile Thr Gly Val Ile Ser Thr Ile Leu Gln Tyr Ser Lys Gln Ala Met Phe Glu His Val Ala Asn Lys Ile His>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>
        1180      1190      1200      1210      1220      1230      1240      1250      1260
          *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
AAC AAA ATT GTA GAA TGG GAA AAA AAT AAT CAC GGT AAG AAC TAC TTT GAA AAT GGT TAC GAT GCC CGT TAT CTT GCG AAT TTA CAA GAT
TTG TTT TAA CAT CTT ACC CTT TTT TTA TTA GTG CCA TTC TTG ATG AAA CTT TTA CCA ATG CTA CGG GCA ATA GAA CGC TTA AAT GTT CTA
Asn Lys Ile Val Glu Trp Glu Lys Asn Asn His Gly Lys Asn Tyr Phe Glu Asn Gly Tyr Asp Ala Arg Tyr Leu Ala Asn Leu Gln Asp>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>
```

FIG.5–5

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      1270      1280      1290      1300      1310      1320      1330      1340      1350
   *    #    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
AAT ATG AAA TTC TTA CTG AAC TTA AAC AAA GAG TTA CAG GCA GAA CGT GTC ATC GCT ATT ACT CAG CAG CAA TGG GAT AAC AAC ATT GGT
TTA TAC TTT AAG AAT GAC TTG AAT TTG TTT CTC AAT GTC CGT CTT GCA CAG TAG CGA TAA TGA GTC GTC GTT ACC CTA TTG TTG TAA CCA
Asn Met Lys Phe Leu Leu Asn Leu Asn Lys Glu Leu Gln Ala Glu Arg Val Ile Ala Ile Thr Gln Gln Gln Trp Asp Asn Asn Ile Gly>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

      1360      1370      1380      1390      1400      1410      1420      1430      1440
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GAT TTA GCT GGT ATT AGC CGT TTA GGT GAA AAA GTC CTT AGT GGT AAA GCC TAT GTG GAT GCG TTT GAA GAA GGC AAA CAC ATT AAA GCC
CTA AAT CGA CCA TAA TCG GCA AAT CCA CTT TTT CAG GAA TCA CCA TTT CGG ATA CAC CTA CGC AAA CTT CTT CCG TTT GTG TAA TTT CGG
Asp Leu Ala Gly Ile Ser Arg Leu Gly Glu Lys Val Leu Ser Gly Lys Ala Tyr Val Asp Ala Phe Glu Glu Gly Lys His Ile Lys Ala>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

      1450      1460      1470      1480      1490      1500      1510      1520      1530
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GAT AAA TTA GTA CAG TTG GAT TCG GCA AAC GGT ATT ATT GAT GTG AGT AAT TCG GGT AAA GCG AAA ACT CAG CAT ATC TTA TTC AGA ACG
CTA TTT AAT CAT GTC AAC CTA AGC CGT TTG CCA TAA TAA CTA CAC TCA TTA AGC CCA TTT CGC TTT TGA GTC GTA TAG AAT AAG TCT TGC
Asp Lys Leu Val Gln Leu Asp Ser Ala Asn Gly Ile Ile Asp Val Ser Asn Ser Gly Lys Ala Lys Thr Gln His Ile Leu Phe Arg Thr>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>
```

FIG.5-6

```
1540          1550          1560          1570          1580          1590          1600          1610          1620
  *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
CCA TTA TTG ACG CCG GGA ACA GAG CAT CGT GAA CGC GTA CAA ACA GGT AAA TAT GAA TAT ATT ACC AAG CTC AAT ATT AAC CGT GTA GAT
GGT AAT AAC TGC GGC CCT TGT CTC GTA GCA CTT GCG CAT GTT TGT CCA TTT ATA CTT ATA TAA TGG TTC GAG TTA TAA TTG GCA CAT CTA
Pro Leu Leu Thr Pro Gly Thr Glu His Arg Glu Arg Val Gln Thr Gly Lys Tyr Glu Tyr Ile Thr Lys Leu Asn Ile Asn Arg Val Asp>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

      1630          1640          1650          1660          1670          1680          1690          1700          1710
  *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
AGC TGG AAA ATT ACA GAT GGT GCA GCA AGT TCT ACC TTT GAT TTA ACT AAC GTT GTT CAG CGT ATT GGT ATT GAA TTA GAC AAT GCT GGA
TCG ACC TTT TAA TGT CTA CCA CGT CGT TCA AGA TGG AAA CTA AAT TGA TTG CAA CAA GTC GCA TAA CCA TAA CTT AAT CTG TTA CGA CCT
Ser Trp Lys Ile Thr Asp Gly Ala Ala Ser Ser Thr Phe Asp Leu Thr Asn Val Val Gln Arg Ile Gly Ile Glu Leu Asp Asn Ala Gly>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

      1720          1730          1740          1750          1760          1770          1780          1790          1800
  *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
AAT GTA ACT AAA ACC AAA GAA ACA AAA ATT ATT GCC AAA CTT GGT GAA GGT GAT GAC AAC GTA TTT GTT GGT TCT GGT ACG ACG GAA ATT
TTA CAT TGA TTT TGG TTT CTT TGT TTT TAA TAA CGG TTT GAA CCA CTT CCA CTA CTG TTG CAT AAA CAA CCA AGA CCA TGC TGC CTT TAA
Asn Val Thr Lys Thr Lys Glu Thr Lys Ile Ile Ala Lys Leu Gly Glu Gly Asp Asp Asn Val Phe Val Gly Ser Gly Thr Thr Glu Ile>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

      1810          1820          1830          1840          1850          1860          1870          1880          1890
  *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
GAT GGC GGT GAA GGT TAC GAC CGA GTT CAC TAT AGC CGT GGA AAC TAT GGT GCT TTA ACT ATT GAT GCA ACC AAA GAG ACC GAG CAA GGT
CTA CCG CCA CTT CCA ATG CTG GCT CAA GTG ATA TCG GCA CCT TTG ATA CCA CGA AAT TGA TAA CTA CGT TGG TTT CTC TGG CTC GTT CCA
Asp Gly Gly Glu Gly Tyr Asp Arg Val His Tyr Ser Arg Gly Asn Tyr Gly Ala Leu Thr Ile Asp Ala Thr Lys Glu Thr Glu Gln Gly>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>
```

FIG.5-7

```
    1900              1910              1920                1930              1940              1950              1960              1970              1980
     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
AGT TAT ACC GTA AAT CGT TTC GTA GAA ACC GGT AAA GCA CTA CAC GAA GTG ACT TCA ACC CAT ACC GCA TTA GTG GGC AAC CGT GAA GAA
TCA ATA TGG CAT TTA GCA AAG CAT CTT TGG CCA TTT CGT GAT GTG CTT CAC TGA AGT TGG GTA TGG CGT AAT CAC CCG TTG GCA CTT CTT
Ser Tyr Thr Val Asn Arg Phe Val Glu Thr Gly Lys Ala Leu His Glu Val Thr Ser Thr His Thr Ala Leu Val Gly Asn Arg Glu Glu>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

         1990              2000              2010                2020              2030              2040              2050              2060              2070
     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
AAA ATA GAA TAT CGT CAT AGC AAT AAC CAG CAC CAT GCC GGT TAT TAC ACC AAA GAT ACC TTG AAA GCT GTT GAA GAA ATT ATC GGT ACA
TTT TAT CTT ATA GCA GTA TCG TTA TTG GTC GTG GTA CGG CCA ATA ATG TGG TTT CTA TGG AAC TTT CGA CAA CTT CTT TAA TAG CCA TGT
Lys Ile Glu Tyr Arg His Ser Asn Asn Gln His His Ala Gly Tyr Tyr Thr Lys Asp Thr Leu Lys Ala Val Glu Glu Ile Ile Gly Thr>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

         2080              2090              2100                2110              2120              2130              2140              2150              2160
     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *     *
TCA CAT AAC GAT ATC TTT AAA GGT AGT AAG TTC AAT GAT GCC TTT AAC GGT GGT GAT GGT GTC GAT ACT ATT GAC GGT AAC GAC GGC AAT
AGT GTA TTG CTA TAG AAA TTT CCA TCA TTC AAG TTA CTA CGG AAA TTG CCA CCA CTA CCA CAG CTA TGA TAA CTG CCA TTG CTG CCG TTA
Ser His Asn Asp Ile Phe Lys Gly Ser Lys Phe Asn Asp Ala Phe Asn Gly Gly Asp Gly Val Asp Thr Ile Asp Gly Asn Asp Gly Asn>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>
```

FIG.5—8

```
2170           2180           2190           2200           2210           2220           2230           2240           2250
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GAC CGC TTA TTT GGT GGT AAA GGC GAT GAT ATT CTC GAT GGT GGA AAT GGT GAT GAT TTT ATC GAT GGC GGT AAA GGC AAC GAC CTA TTA
CTG GCG AAT AAA CCA CCA TTT CCG CTA CTA TAA GAG CTA CCA CCT TTA CCA CTA CTA AAA TAG CTA CCG CCA TTT CCG TTG CTG GAT AAT
Asp Arg Leu Phe Gly Gly Lys Gly Asp Asp Ile Leu Asp Gly Gly Asn Gly Asp Asp Phe Ile Asp Gly Gly Lys Gly Asn Asp Leu Leu>
---c---c---c---c---c---c---c---c---c---c----RECOMBINANT LEUKOTOXIN PEPTIDE_c---c---c---c---c---c---c---c---c---c---c--->

     2260           2270           2280           2290           2300           2310           2320           2330           2340
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CAC GGT GGC AAG GGC GAT GAT ATT TTC GTT CAC CGT AAA GGC GAT GGT AAT GAT ATT ATT ACC GAT TCT GAC GGC AAT GAT AAA TTA TCA
GTG CCA CCG TTC CCG CTA CTA TAA AAG CAA GTG GCA TTT CCG CTA CCA TTA CTA TAA TAA TGG CTA AGA CTG CCG TTA CTA TTT AAT AGT
His Gly Gly Lys Gly Asp Asp Ile Phe Val His Arg Lys Gly Asp Gly Asn Asp Ile Ile Thr Asp Ser Asp Gly Asn Asp Lys Leu Ser>
---c---c---c---c---c---c---c---c---c---c----RECOMBINANT LEUKOTOXIN PEPTIDE_c---c---c---c---c---c---c---c---c---c---c--->

     2350           2360           2370           2380           2390           2400           2410           2420           2430
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
TTC TCT GAT TCG AAC TTA AAA GAT TTA ACA TTT GAA AAA GTT AAA CAT AAT CTT GTC ATC ACG AAT AGC AAA AAA GAG AAA GTG ACC ATT
AAG AGA CTA AGC TTG AAT TTT CTA AAT TGT AAA CTT TTT CAA TTT GTA TTA GAA CAG TAG TGC TTA TCG TTT TTT CTC TTT CAC TGG TAA
Phe Ser Asp Ser Asn Leu Lys Asp Leu Thr Phe Glu Lys Val Lys His Asn Leu Val Ile Thr Asn Ser Lys Lys Glu Lys Val Thr Ile>
---c---c---c---c---c---c---c---c---c---c----RECOMBINANT LEUKOTOXIN PEPTIDE_c---c---c---c---c---c---c---c---c---c---c--->

     2440           2450           2460           2470           2480           2490           2500           2510           2520
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CAA AAC TGG TTC CGA GAG GCT GAT TTT GCT AAA GAA GTG CCT AAT TAT AAA GCA ACT AAA GAT GAG AAA ATC GAA GAA ATC ATC GGT CAA
GTT TTG ACC AAG GCT CTC CGA CTA AAA CGA TTT CTT CAC GGA TTA ATA TTT CGT TGA TTT CTA CTC TTT TAG CTT CTT TAG TAG CCA GTT
Gln Asn Trp Phe Arg Glu Ala Asp Phe Ala Lys Glu Val Pro Asn Tyr Lys Ala Thr Lys Asp Glu Lys Ile Glu Glu Ile Ile Gly Gln>
---c---c---c---c---c---c---c---c---c---c----RECOMBINANT LEUKOTOXIN PEPTIDE_c---c---c---c---c---c---c---c---c---c---c--->
```

FIG.5—9

```
 2530      2540      2550      2560      2570      2580      2590      2600      2610
    *         *         *         *         *         *         *         *         *
AAT GGC GAG CGG ATC ACC TCA AAG CAA GTT GAT GAT CTT ATC GCA AAA GGT AAC GGC AAA ATT ACC CAA GAT GAG CTA TCA AAA GTT GTT
TTA CCG CTC GCC TAG TGG AGT TTC GTT CAA CTA CTA GAA TAG CGT TTT CCA TTG CCG TTT TAA TGG GTT CTA CTC GAT AGT TTT CAA CAA
Asn Gly Glu Arg Ile Thr Ser Lys Gln Val Asp Asp Leu Ile Ala Lys Gly Asn Gly Lys Ile Thr Gln Asp Glu Leu Ser Lys Val Val>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

      2620      2630      2640      2650      2660      2670      2680      2690      2700
         *         *         *         *         *         *         *         *         *
GAT AAC TAT GAA TTG CTC AAA CAT AGC AAA AAT GTG ACA AAC AGC TTA GAT AAG TTA ATC TCA TCT GTA AGT GCA TTT ACC TCG TCT AAT
CTA TTG ATA CTT AAC GAG TTT GTA TCG TTT TTA CAC TGT TTG TCG AAT CTA TTC AAT TAG AGT AGA CAT TCA CGT AAA TGG AGC AGA TTA
Asp Asn Tyr Glu Leu Leu Lys His Ser Lys Asn Val Thr Asn Ser Leu Asp Lys Leu Ile Ser Ser Val Ser Ala Phe Thr Ser Ser Asn>
___c___c___c___c___c___c___c___c___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE_c___c___c___c___c___c___c___c___c___c___c___>

      2710      2720      2730      2740      2750      2760      2770      2780      2790
         *         *         *         *         *         *         *         *         *
GAT TCG AGA AAT GTA TTA GTG GCT CCA ACT TCA ATG TTG GAT CAA AGT TTA TCT TCT CTT CAA TTT GCT AGG GGA TCC TAG CTAGCTAGCCATG
CTA AGC TCT TTA CAT AAT CAC CGA GGT TGA AGT TAC AAC CTA GTT TCA AAT AGA AGA GAA GTT AAA CGA TCC CCT AGG ATC GATCGATCGGTAC
Asp Ser Arg Asn Val Leu Val Ala Pro Thr Ser Met Leu Asp Gln Ser Leu Ser Ser Leu Gln Phe Ala Arg Gly Ser End>
___c___c___c___c___c___c___c___c___c__RECOMBINANT LEUKOTOXIN PEPTIDE___c___c___c___c___c___c___c___c___c___>
                                                                                   _b___VECTOR SEQUENCE_______>

*
G
C
_>
```

FIG.6A

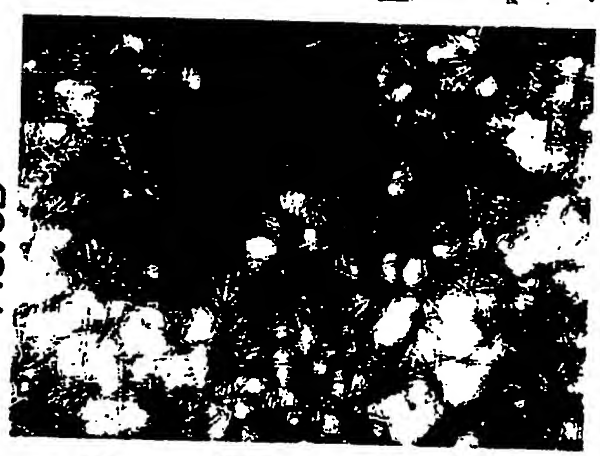


FIG.6B

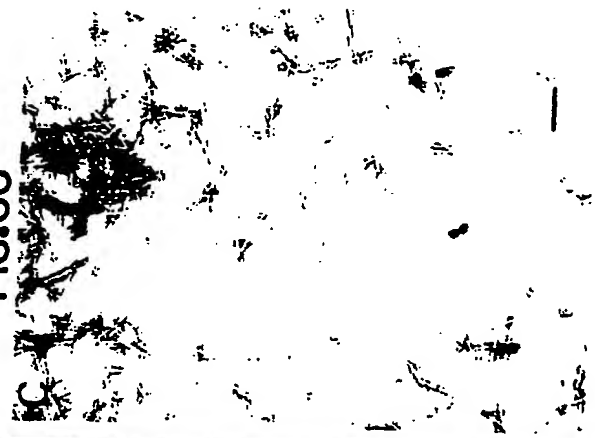
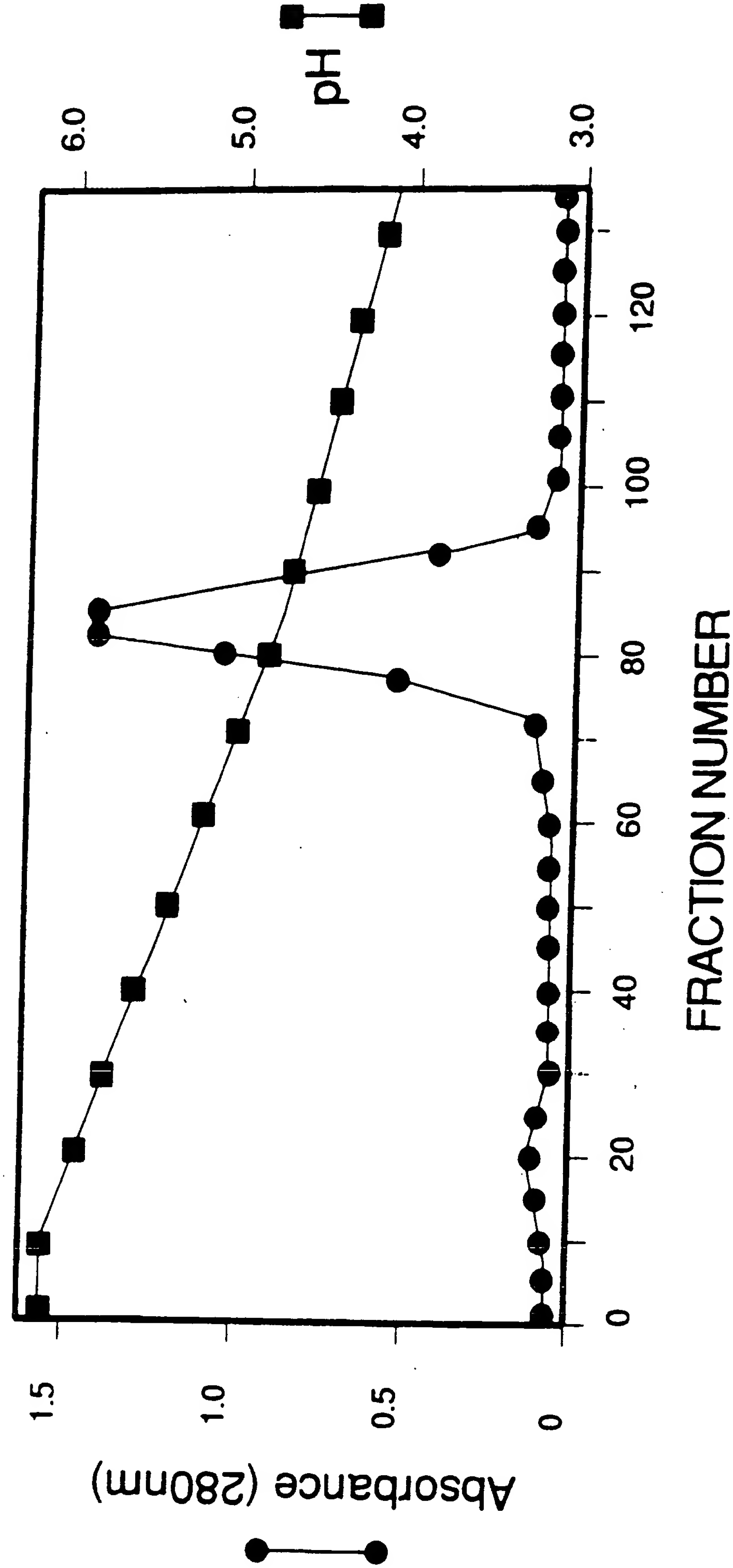


FIG.6C

FIG. 7


pH
6.0
5.0
4.0
3.0
Absorbance (280nm)
1.5
1.0
0.5
0
0
20
40
60
80
100
120
FRACTION NUMBER

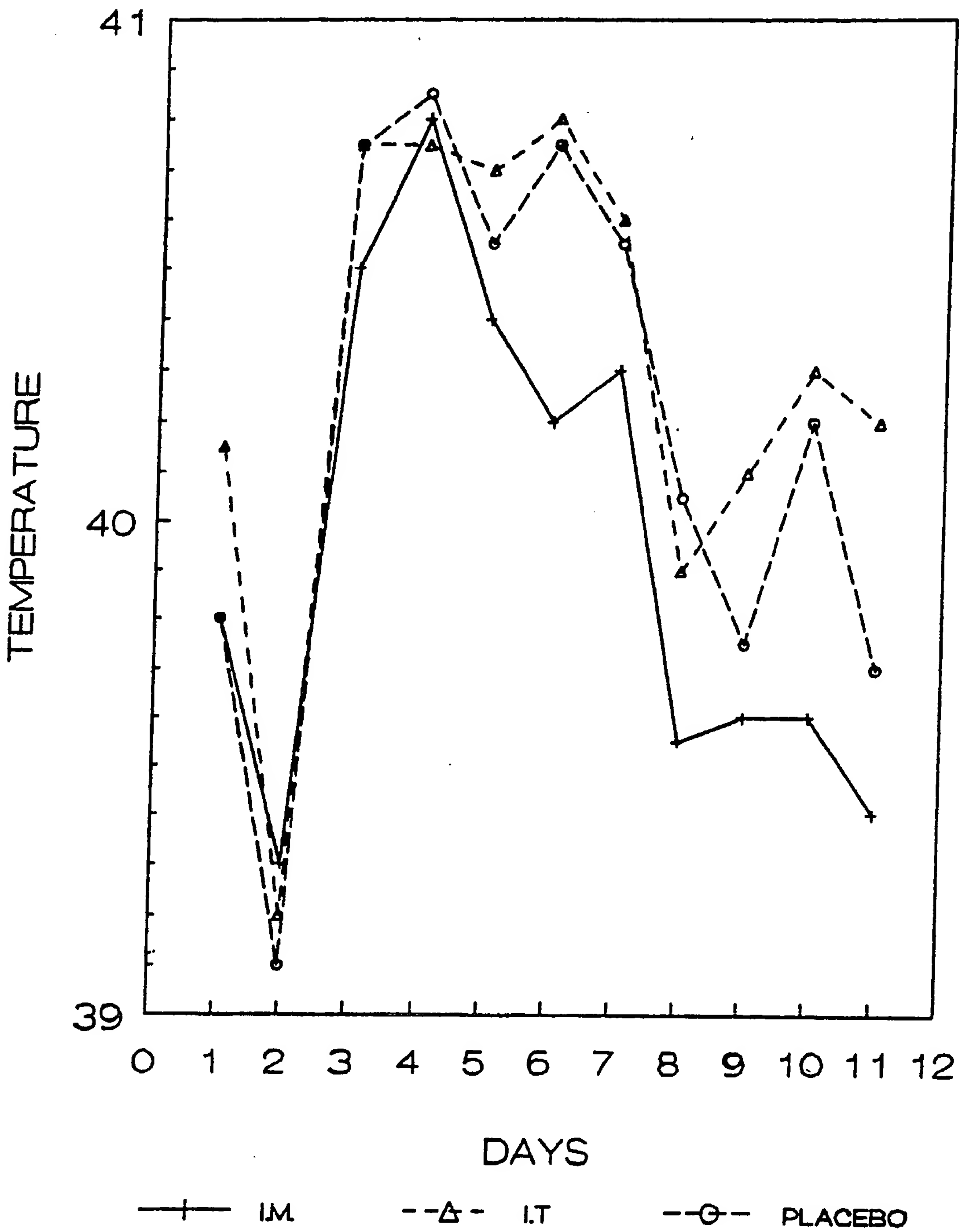
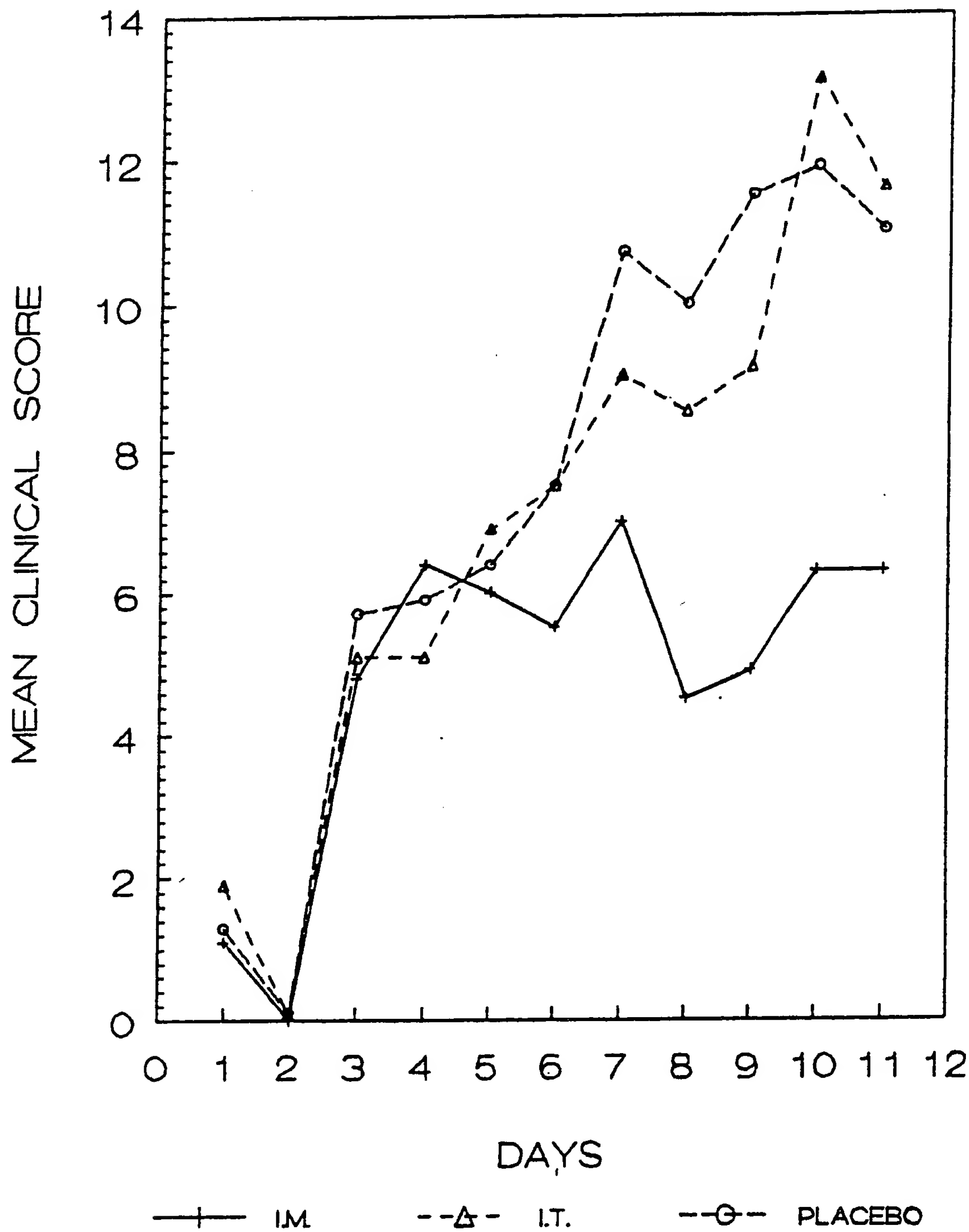


FIG. 8



14
12
10
8
6
4
2
0
MEAN CLINICAL SCORE
0 1 2 3 4 5 6 7 8 9 10 11 12
DAYS
I.M.
I.T.
PLACEBO

FIG. 9

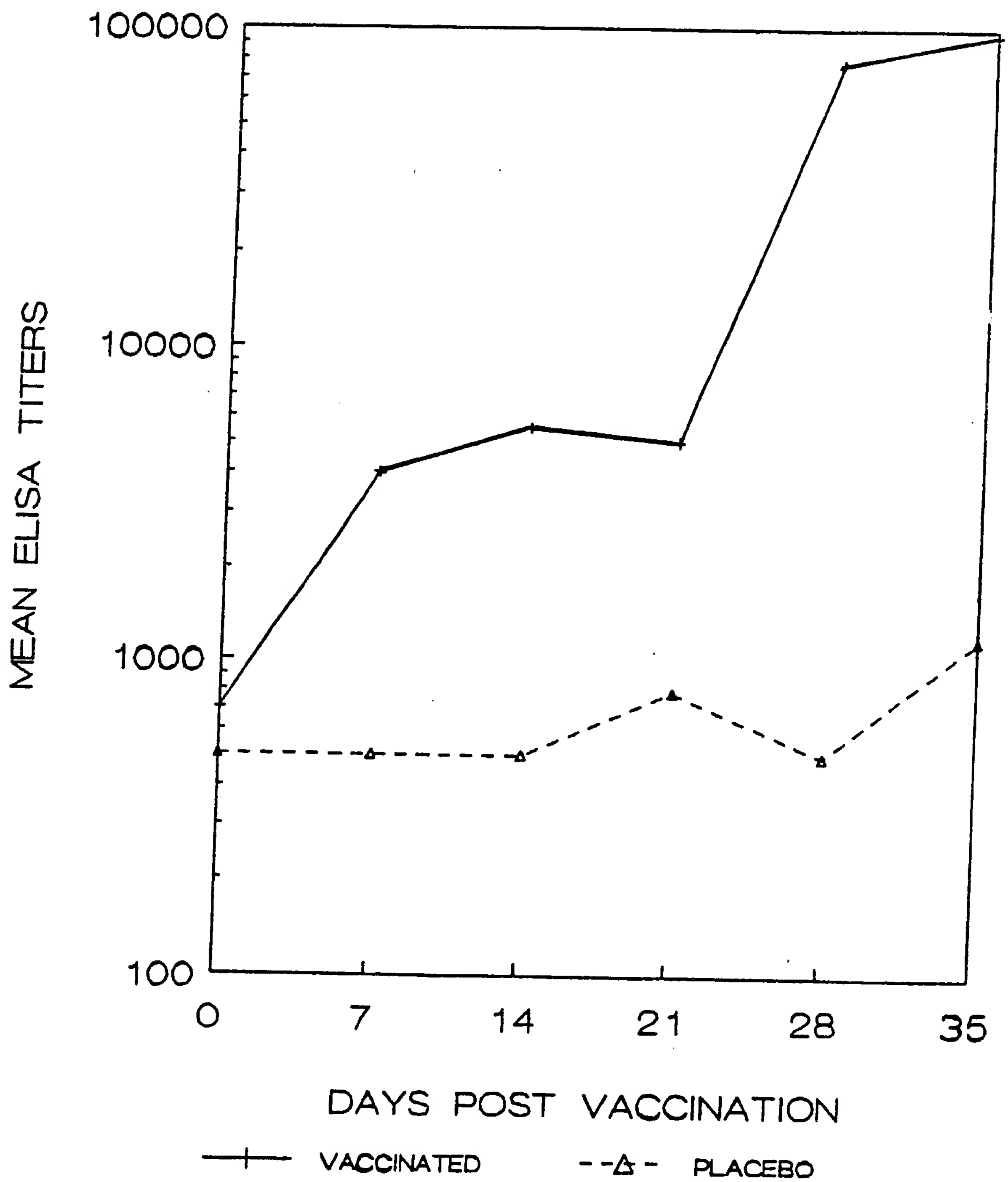
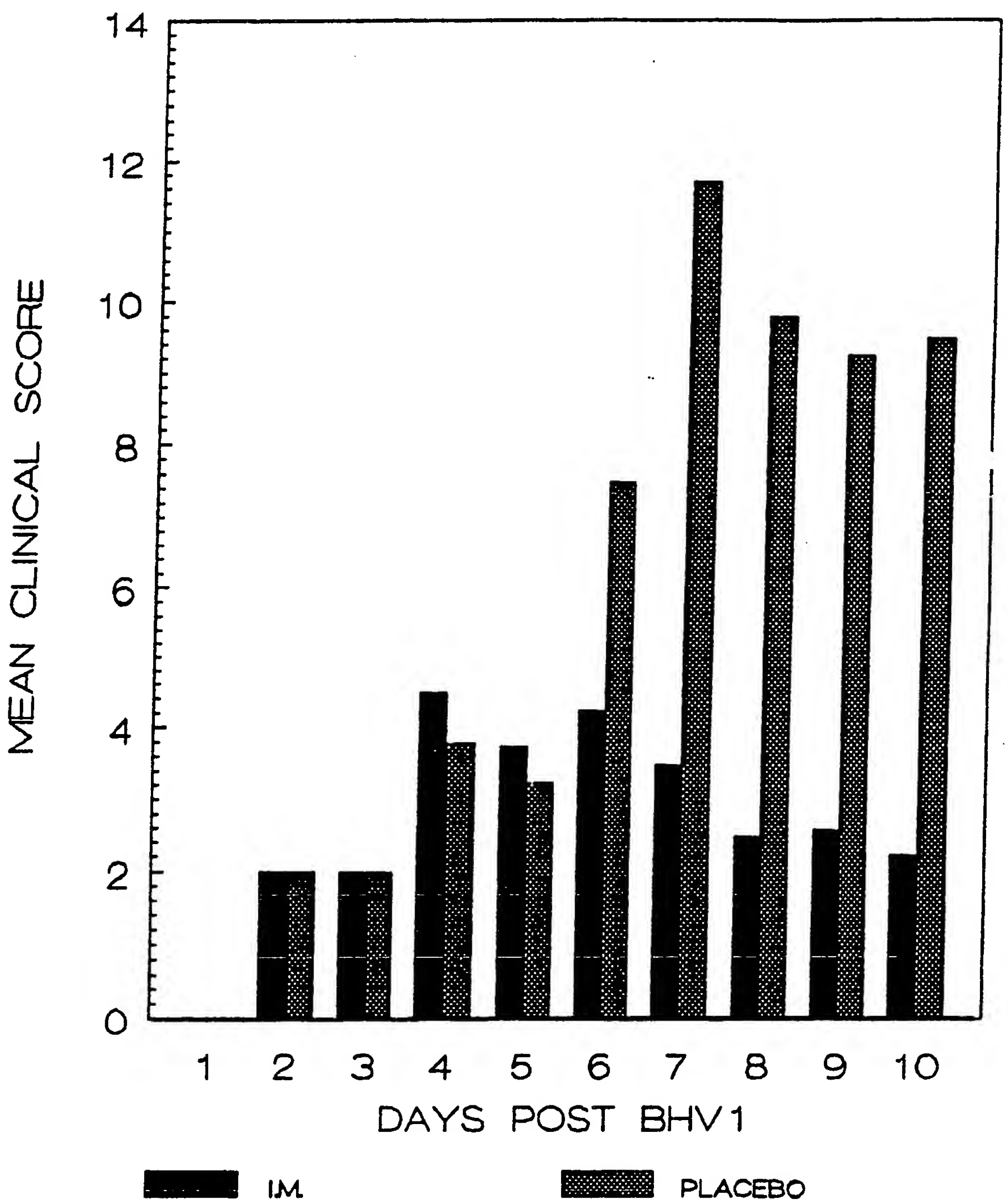

100000
10000
1000
100
MEAN ELISA TITERS
0
7
14
21
28
35
DAYS POST VACCINATION
VACCINATED
PLACEBO

FIG. 10



14
12
10
8
6
4
2
0
MEAN CLINICAL SCORE
1
2
3
4
5
6
7
8
9
10
DAYS POST BHV1
I.M.
PLACEBO

FIG. 11

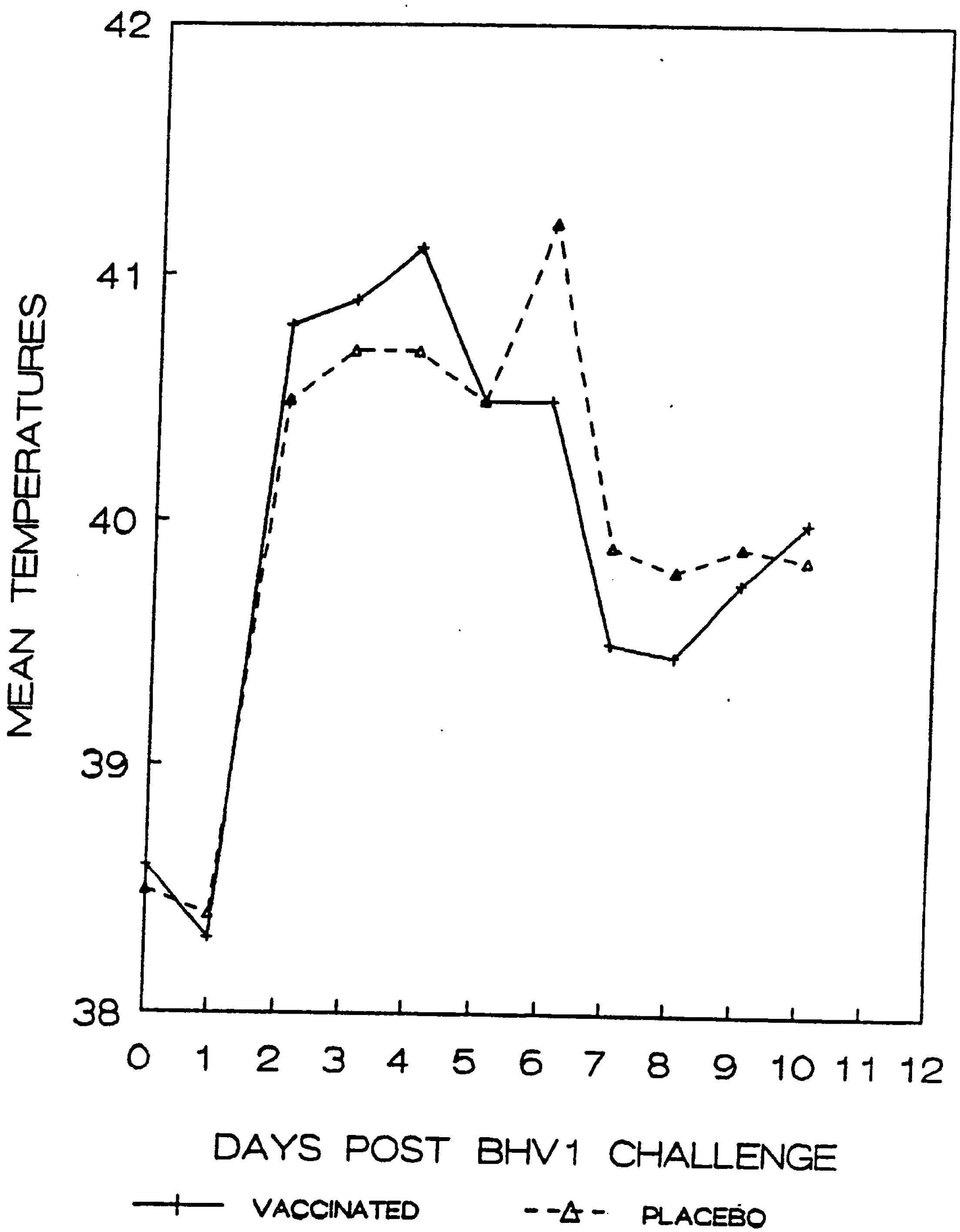
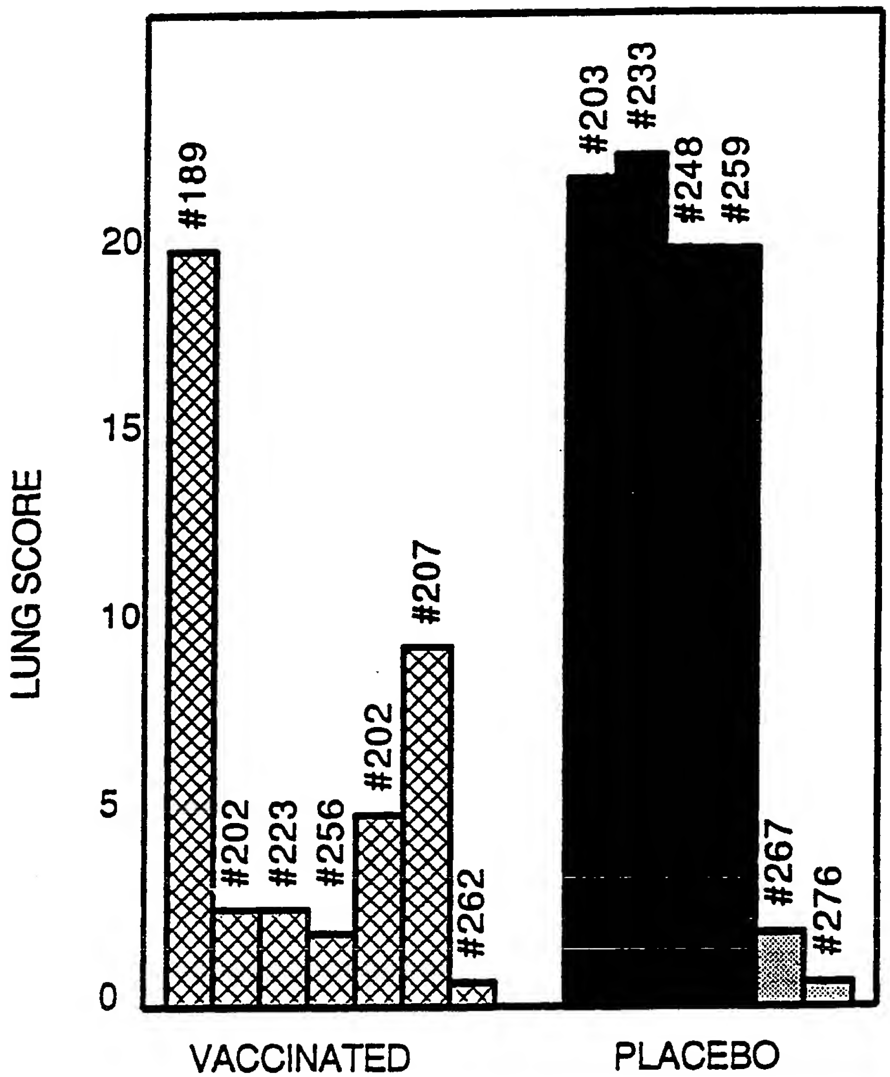

42
41
40
39
38
MEAN TEMPERATURES
0 1 2 3 4 5 6 7 8 9 10 11 12
DAYS POST BHV1 CHALLENGE
VACCINATED
PLACEBO

FIG. 12



LUNG SCORE
0
5
10
15
20
#189
#202
#223
#256
#202
#207
#262
#203
#233
#248
#259
#267
#276
VACCINATED
PLACEBO

FIG. 13

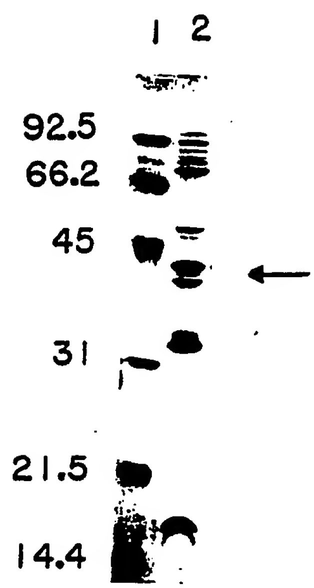


FIG. 14

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 90/00170

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^5: A 61 K 39/102, C 12 N 15/31, A 61 K 39/395

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^5	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	Biological Abstracts, vol. 79, no. 2, 1985, Biological Abstracts Inc., (Philadelphia, US), P.G. Squire et al.: "Identification and extraction of Pasteurella haemolytica membrane proteins", see abstract 16349, & Infect. Immun. 45(3), 667-673, 1984 --	
A	Biological Abstracts, vol. 85, no. 9, 1988, Biological Abstracts Inc., (Philadelphia, US), P.E. Shewen et al.: "Vaccination of calves with leukotoxic culture super-natant from Pasteurella haemolytica", see abstract 90225, & Can. J. Vet. Res. 52(1), 30-36, 1988 ----------	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
6th December 1990	21. 12. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	miss T. MORTENSEN